ANNUAL NEWSLETTER - N°21 MARCH 2023

Labinfo

Newsletter for the approved food safety laboratories

Responsible editor: Herman Diricks

FASFC
AC-Kruidtuin - Food Safety Center
Kruidtuinlaan 55,
1000 Brussel

NRL NATIONAL REFERENCE LABORATORIES

LabInfo
Newsletter for the approved food safety laboratories

Editors' group
L. Bollen, R. Vermeylen, G. Janssens, E. Willem

Authors of this issue
Y. Ghafir, P. Szternfeld, V. Van Leeuw, L. Joly, M. De Proft, T. J. McGrath, F. Limonier, G. Poma, J. Bombeke, R. Winand, K. Vanneste, M. Andjelkovic, E. Van Hoeck, A. Covaci, H. Demaegdt, N. Waegeneers, K. Cheyns, A. Ruttens, S. Goscinny, H. Debergh, M. C. Garcia Graells, B. Verhaegen, K. Van Hoorde, M-A. Fraiture, N. Papazova, N. Roosens, S. V. Malysheva and J. Masquelier

Translation
Translation Service of the Agency
Editors' group

Photographs and illustrations
Supplied by the laboratories

Layout
Communicatiedienst FAVV

Editor's address
LabInfo
p.a. L. Bollen
FASFC
AC-Kruidtuin – Food Safety Center
4de verdieping, bureel K04/120220
Kruidtuinlaan 55
1000 Brussel
Tel.: 02.211.87.34
luc.bollen@afsca-favv.be

Labinfo Editorial

Dear Reader,

Here is the 21st edition of Labinfo for 2023.

In 2022 the situation with regard to the coronavirus health crisis has improved and we are almost back to normal. The Agency has taken this opportunity to adapt its way of working, particularly with regard to the use of video-conferencing and teleworking. Like every year, there were a few incidents, including Salmonella contaminations of chocolate and several outbreaks of avian influenza. As in previous years, however, the Agency could count on the support and cooperation of the NRLs and other approved laboratories. I would therefore like to reiterate my sincere thanks to all the parties involved for their cooperation.

At the beginning of 2021, the Director-General of the Laboratories Administration left the FASFC and he has not been replaced since. Together with Mandy Lekens, Director of Internal Laboratories, we have taken over his various tasks as best as we could.

The current geopolitical context and the savings imposed on the federal administrations, including the FASFC, have also had an impact and may lead in the future to a reduction in the number of samples taken and consequently also in the number of analyses to be carried out.

The articles published in this edition cover a wide variety of topics, such as pesticides in tea, marine toxins, authenticity testing, allergens, GMOs and, last but not least, plant toxins.

I hope you will enjoy reading this new edition of Labinfo.

Yasmine Ghafir
Director of External Laboratories

Are pesticides transferred in your tea?

Philippe Szternfeld[1], Claire Demoury[2], Laure Joly[1]

Sciensano
[1]Organic Contaminants and Additives
[2]Risk and Health Impact Assessment
Rue Juliette Wytsman 14, 1050 Brussel
Philippe.szternfeld@sciensano.be

Background

Controls performed by the Belgian Federal Agency for Safety of the Food Chain (FASFC) show that tea and herbal tea very often contained pesticides residues. In some cases concentrations found exceed the maximum residue limits (MRLs) fixed by European regulations. Between 2017 and 2019, more than 90 different residues were detected in these samples. The most commonly found residues by frequency of detection are: anthraquinone, bifenthrin, cypermethrin, thiamethoxam, acetamiprid, cyhalothrin-λ, thiacloprid, carbendazim, chlorfenapyr, chlorpyrifos, imidacloprid, propargite, 2,4-D, tolfenpyrad and dinotefuran. When the residue content exceeds the MRL, the tea or herbal tea cannot be put on the market nor used and the FASFC performs a risk assessment in order to define the extent of measures to be taken. However, a risk assessment must take into account that the residues are analysed in the tea leaves whereas it is the brewed tea that is being consumed. Therefore, transfer factors (TF), also called brewing factors, which account for this infusion process should be used. However, no TF are available for several residues detected in tea or infusions on the Belgian market. This study aims to address this issue by creating a TF database for tea infusion that can be used as a tool for competent authorities (FASFC) when performing a risk assessment.

To carry out this project, a strategy consisting of 3 main axes was followed: (i) scientific literature and existing databases were reviewed in order to select relevant TF for Belgium (type of tea and similar infusion parameters); (ii) Experimental determination of missing TF; (iii) Development of a predictive model for TF based on their physicochemical properties. All TF considered relevant from scientific literature or existing databases and experimentally determined, and predictive models were compiled into one database. This database contains 1206 TF for 108 pesticides for different types of tea (black, green, herbal, aromatized tea). This article focuses on the experimental determination of missing TF and the predictive model elaboration.

Experimental determination of transfer factors

The experimental determination of TF requires a tea that is contaminated with the pesticides of interest. Moreover the contamination should be high enough as pesticide residues are expected to be highly diluted during infusion. Unfortunately, teas sufficiently contaminated for experimental TF determination, were not available on the Belgian market for all pesticides. Hence, as an alternative approach organic teas were purchased in local markets and spiked with a solution containing 54 pesticides, most frequently found on the Belgian market. The preparation protocol for pesticide analysis is summarized in Figure 1.

Figure 1. Preparation protocol for the analysis of pesticides in tea brew

Based on pesticide residue concentration analysed in tea leaves and brewed tea, transfer factors have been determined according to following formula:

$$\text{Transfer factor (\%)} = \frac{quantity\ of\ pesticides\ (tea\ brew)}{quantity\ of\ pesticides\ (tea\ leaves)} \text{ X } 100\%$$

Parameters influencing the transfer of pesticides

The influence of some parameters (tea type, infusion time, flavours, water hardness) on the TF has been assessed with a Mann Whitney statistical test with a 99 % confidence level (p-value = 0.01). The impact of the different parameters is illustrated for the two pesticide classes most frequently found in tea leaves, namely pyrethroids and neonicotinoids.

INFUSION TIME

The impact of the infusion time on TF was assessed for 2, 3, 5 and 10 min of infusion, in order to take into account the difference of consumer behaviour (Figure 2). Two important conclusions could be drawn. Firstly, the infusion time does not play a major role in the transfer of pesticides. This has been confirmed for all 54 studied pesticides. Therefore time infusion does not need to be considered during model development and risk assessment. Secondly neonicotinoids (N) are strongly transferred to the brew (50-100 %), which is not the case for pyrethroids (P) (<10 % for cyhalothrin-λ, cypermethrin, permethrin). This phenomenon can be explained by the fact that pyrethroids have a very low affinity for water (polarity, log P > 6).available.

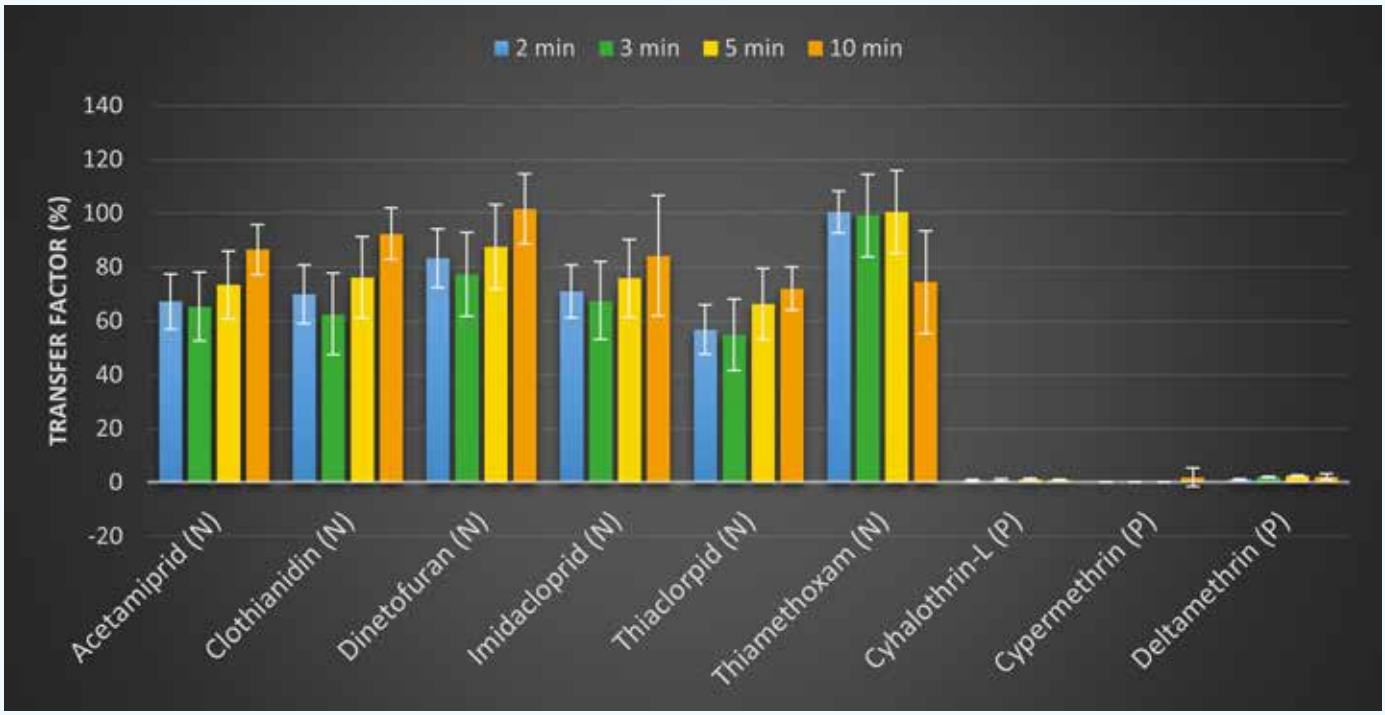


Figure 2. transfer factor for some neonicotinoid (N) and pyrethroid (P) pesticides depending on infusion time (2, 3, 5 and 10 min)

TYPE OF TEA

The behaviour of pesticides according the type of tea has been assessed with black tea and green tea, the two most consumed tea types in Belgium. For about half of the studied pesticides (29/54), a statistically significant difference in transfer according to the type of tea was found. In most cases a lower transfer rate was observed for green tea. Figure 3 illustrates the behaviour for some pesticides according to the type of tea. TF of thiacloprid and thiamethoxam are much lower for green tea compared to black tea, although this difference was not observed for the other neonicotinoids.

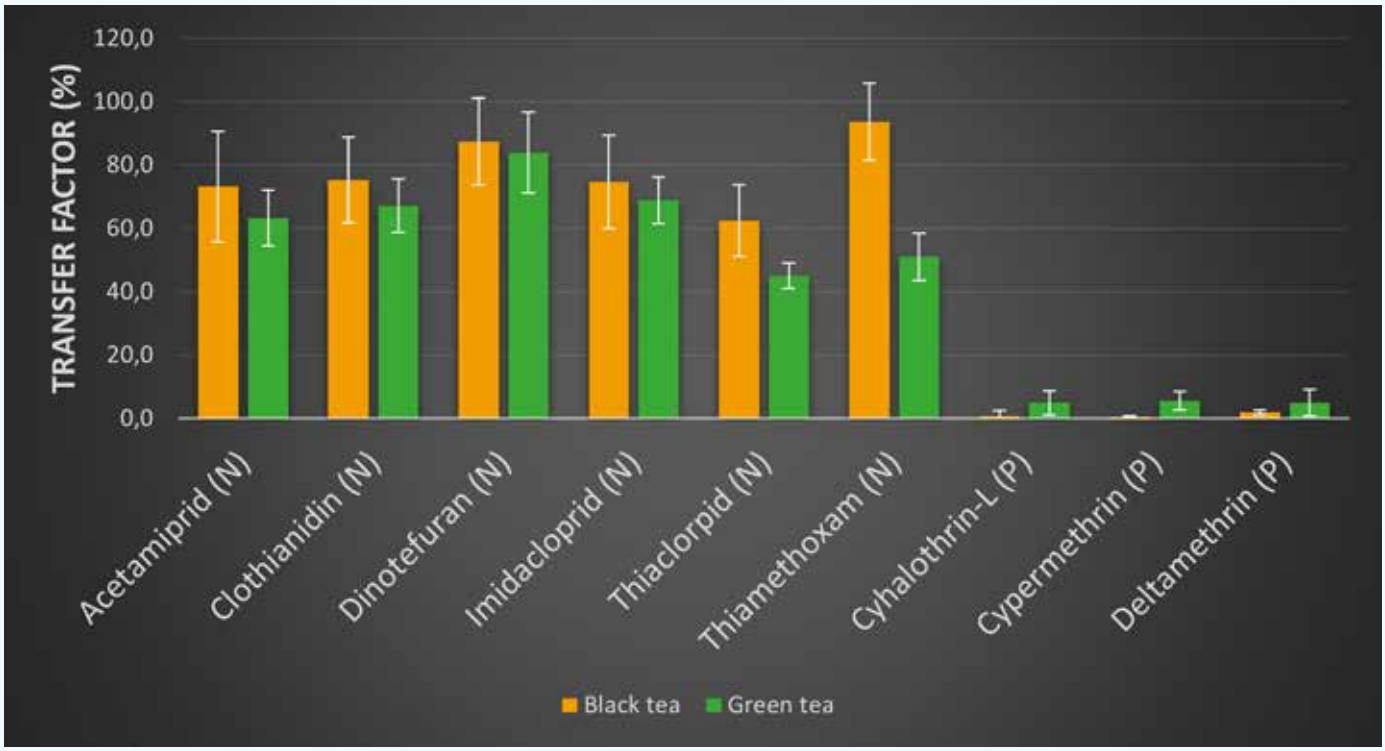


Figure 3. Comparison of pesticides transfer factors in black tea (orange box) and green tea (green box)

Spiked black and green teas were infused at two different temperatures (80 °C and 100 °C). No statistical difference in TF related to infusion temperature has been noticed for the studied pesticides.

FLAVOURED TEA

Pincemaille et al. (2014) [1] suggested that some flavours increase the transfer of some very non-polar compounds such as polycyclic aromatic hydrocarbons. Three green tea perfumed with flavour (mint, lemon and orange) bought on the Belgian market were spiked with pesticides and their TF were compared to green tea without flavour (Figure 4). No significant increase of TF has been noticed for very non-polar pesticides (cypermethrin, cyhalothrin-λ, delatamethrin). For pesticides considered as polar (dinotefuran, thiamethoxam, clothianidin), the TF was lower for green tea with lemon and orange flavour but not for tea with mint. The theory of Pincemaille et al. (2004) has thus not been confirmed for non-polar pesticides. Nevertheless, orange and lemon flavours have a reducing impact on the transfer of 21 and 8 out of 54 pesticides respectively.

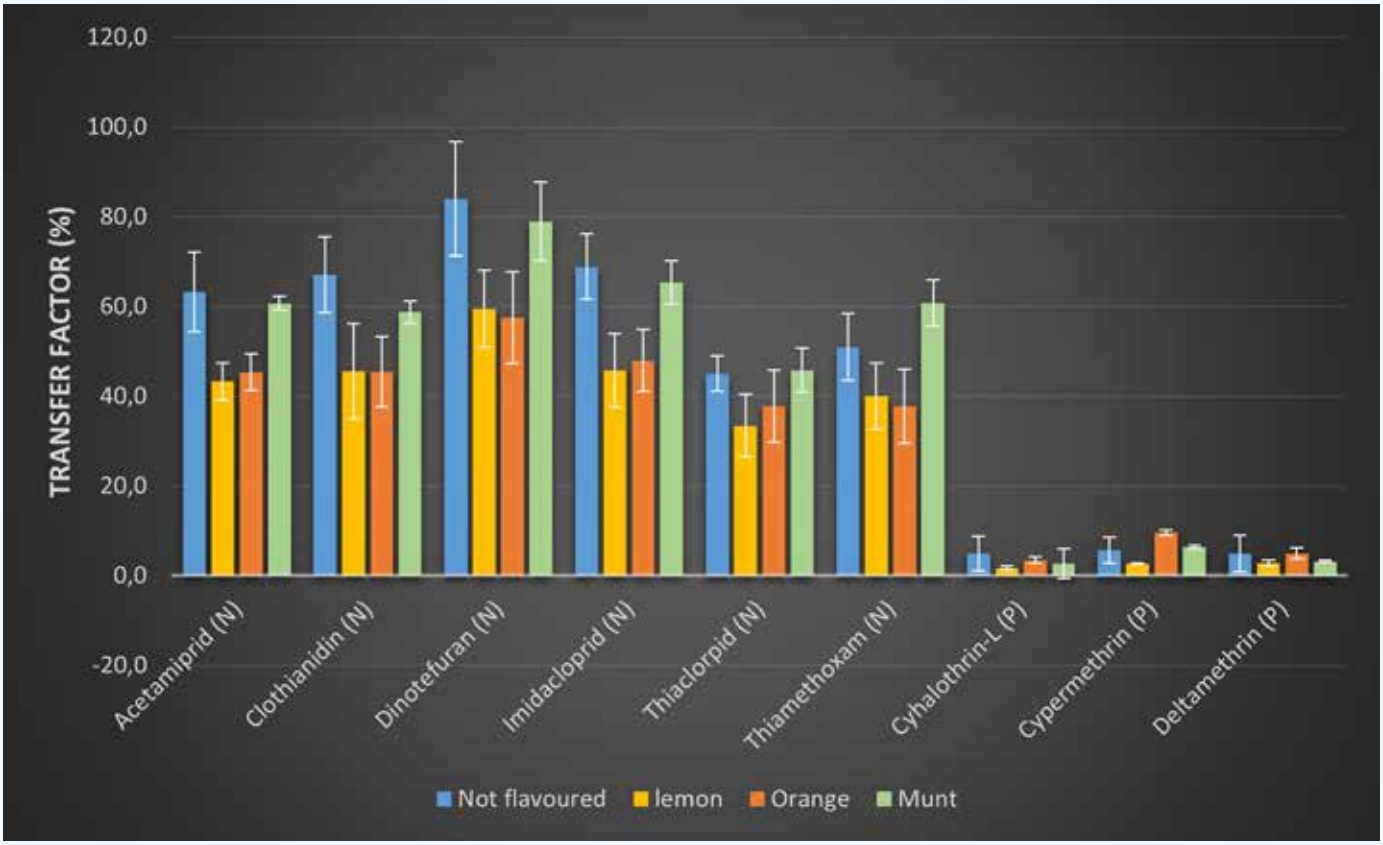


Figure 4. Comparison of transfer rate in flavoured (lemon, orange and munt) and not flavoured green tea

WATER HARDNESS

The influence of water hardness due to the presence of calcium and sodium ions has been assessed by brewing tea with milliQ water (close to 0 °f) and tap water from Brussels which is considered as very hard (38 °f). No statistical difference has been noticed when infusing tea leaves with these two types of water. This conclusion is important in case of risk assessment as this parameter is area dependant and not controllable.

Modelling of pesticides behaviour during infusion

The experimental data were used to predict TF for each type of tea using multivariate log-linear models and considering following physicochemical properties of the pesticides: log P (polarity), solubility (mg/L), and vapour pressure (mPa),:

$$\mathbf{Log(TF) = \beta 0 + \beta 1 \log (P) + \beta 2 \log (vapour\ pressure) + \beta 3 \log (solubility)}$$

These models aim to determine the beta coefficients that minimize the deviations between the experimentally observed and predicted TF. For pesticides with a log P greater than 4, low TF and low variation were observed. Therefore, a fixed TF of maximum 20 % was assumed and are take into account for the elaboration of the predictive model. Log P was the most important variable to describe pesticide transfer into the brew, followed by solubility. Vapour pressure did not give any added value to the modelling. The predicted-by-observed charts of figure 5 display the modelling precision for black tea and green tea.

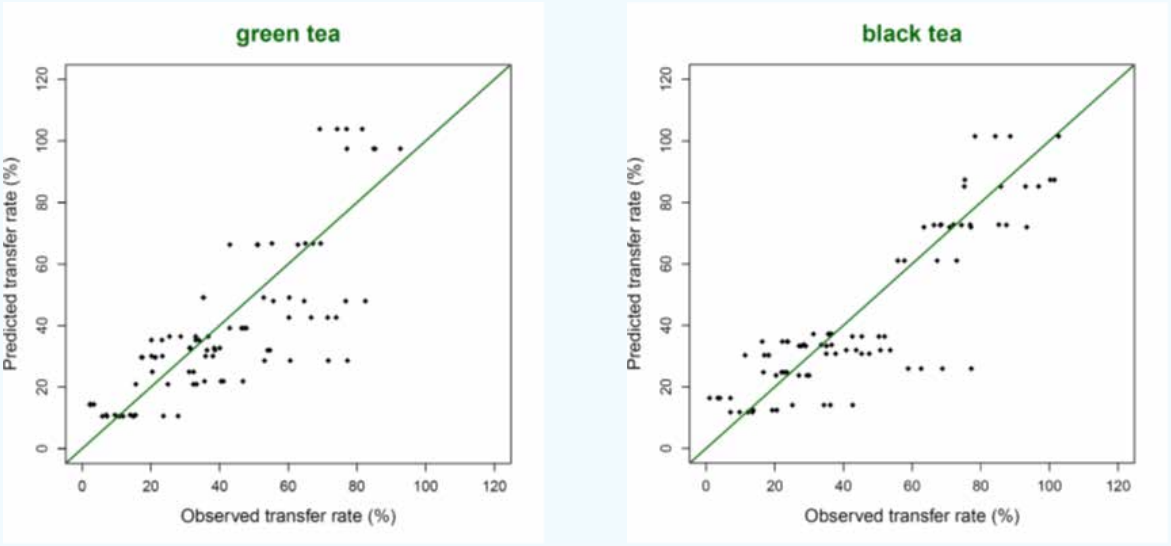


Figure 5. Predicted-by-observed charts for the transfer factors (only for pesticides with log P <4) for 2, 3, 5 and 10 min of infusion for green tea and black tea

These models correctly predict the transfer factors of some pesticides (closed to the green line). For some, they tend to overestimate (above the green line) or underestimate (below the green line) the TF, implying that for the risk assessment of these pesticide residues, preference should be given to experimentally determined TF. Nevertheless, the models offer an acceptable alternative when no data are available for a specific pesticide.

Risk assessment in case of MRL exceedance

To perform a risk assessment in case of MRL exceedance in tea or herbal tea, based on the results presented above it is recommended to use following principles for using the TF compilation database:

1 Apply the maximum experimentally determined TF for the pesticide/tea combination from the database.
2 If no experimental data is available:
   a. Pesticides with Log P > 4: the transfer rate can be estimated at 20 % maximum
   b. Pesticides with Log P < 4: apply the convenient model for each type of tea introducing the different physicochemical parameters of the pesticide.

Conclusion

During this study some infusion parameters that play a major role in the transfer of pesticide residues from tea leaves to brewed tea (type of tea, flavour) have been identified. Interestingly, consumers behaviour (infusion time and temperature (range 80 – 100 °C)) and region depending factors (water hardness) do not play a major role in pesticide transfer. This significantly facilitates the risk assessment as these parameters are difficult to control.

The modelling of pesticides behaviour during tea infusion demonstrates that it is not possible to predict the transfer of all pesticides with adequate precision. It is preferable to refer to a database containing these transfer factors for each pesticide/tea type combination when data exist. Such a database has been created during this study and contains 1206 transfer factors for 108 pesticides (from the scientific literature or laboratory experiments) for different teas and herbal teas frequently consumed by the Belgian population.

Acknowledgments

The research that yielded these results was funded by the Belgian FASFC through the contract for the project "FAVV-AFSCA/STUDIEPROJECT-PROJET D'ETUDE/2020-01: Etude sur des facteurs d'infusion pour les résidus de pesticides présents dans le thé et les infusions".

References

1. Pincemaille J, Schummer C, Heinen E, Moris G (2014) Determination of polycyclic aromatic hydrocarbons in smoked and non-smoked black teas and tea infusions. Food Chemistry 145:807–813. https://doi.org/10.1016/j.foodchem.2013.08.121

Ciguatera Poisoning : an emerging risk in Europe ?

Nathalie Gillard, Aline Vanwynsberghe, Laura Pirlot

Ciguatera (Shell) Fish Poisoning (CP) is the most prevalent phycotoxin-related seafood poisoning across the globe, affecting between 10,000 and 50,000 people annually. This poisoning results from the consumption of fish and shellfish contaminated with toxins produced by dinoflagellates of the genera Gambierdiscus and Fukuyoa and may lead to gastrointestinal (e.g. vomiting, diarrhoea, nausea), neurological (e.g. tingling, itching) and cardiovascular (e.g. hypotension, bradycardia) effects.

Following the ingestion of Gambierdiscus or Fukuyoa cells by herbivorous and omnivorous organisms, toxins are biotransformed and transmitted along the trophic food webs to top-chain and long-living carnivores, including fish and marine mammals. The complex process explaining toxins accumulation, elimination and transfer to carnivores is still poorly understood. While higher-level carnivorous fish generally exhibit greater toxin concentrations than smaller fish and herbivores, this is not always the case[1]. Beside fish and marine mammals, it should also be underlined that sporadic poisoning cases involving octopus, crabs and worm shell have been reported in the Cook Islands[2].

Classification of toxins

The two main toxin groups produced by Gambierdiscus and Fukuyoa dinoflagellates are ciguatoxins (CTXs) and maitotoxins (MTXs).

CTXs are lipophilic toxins and are considered as primarly responsible for CP. These cyclic polyether compounds of around 1100 Da have been classified according to their geographical location as Pacific (P-CTXs), Caribbean (C-CTXs) and Indian (I-CTXs) ciguatoxins.

MTXs are also cyclic polyethers but with an intermediate polarity due to their sulfate ester group(s); their lower intestinal absorption raise doubts in their involvement in CP[3]. Most maitotoxins can be classified depending on their mechanism of action into two groups: MTX-like compounds (MTX1, MTX2, MTX4), associated with a massive Ca^{2+} influx causing a rapid cell death and CTX-like compounds (MTX3, gambierone), which create a disequilibrium in the voltage-gated sodium channels and with much lower potency.

Table 1. Classification of CTXs and other Gambierdiscus metabolites (adapted from FAO 2018).

Compound	Synonyms		Relative potency/ TEF	Available standard
Ciguatoxin 4A group (CTX4A and derivatives)				
CTX1B	CTX, CTX1b, CTX1B, CTX1, P-CTX-1, P-CTX1B	1110.6	1.0	BOC sciences
CTX1A	52-epiCTX, 52-epiCTX1B	1110.6		/
54-deoxyCTX1B	CTX3, P-CTX-3, 4-deoxyCTX, 54-deoxyCTX1B	1094.6	0.2	/
52-epi-54-deoxyCTX1B	CTX-2, P-CTX2, 52-epi-54-deoxyCTX, 52-epi-54-deoxyCTX1B	1094.5	0.2/0.3	/
CTX4B	(gambiertoxin-4B) GTX4B, CTX4B, scaritoxin, P-CTX4B	1060.6	0.05	/
CTX4C	/		/	/
M-seco-CTX4A/4B	M-seco-CTX4A,			
M-seco-CTX4A/4B	1078.6	/	/	
Ciguatoxin 4A group (CTX4A and derivatives)				
7-oxoCTX1B	7-oxoCTX	1126.6	/	/
7-hydroxyCTX1B	7-hydroxyCTX	1128.6	/	/
4-hydroxy-7-oxoCTX1B	4-hydroxy-7-oxoCTX	1144.6	/	/
54-deoxy-50-hydroxyCTX1B	54-deoxy-50hydroxyCTX		/	/
Ciguatoxin 3C group (CTX3C and derivatives)				
CTX3C	CTX3C, P-CTX3C	1022.6	0.2	VWR, Wako
CTX3B	49-epiCTX3C		/	/
51-hydroxyCTX3C	/	1038.6	1.3	/
2,3-dihydro-2,3-dihydroxyCTX3C	2,3,-dihydroxyCTX3C	1056.6	0.1	/
2,3-dihydro-2-hydroxyCTX3C	/	1040.6	/	/
2,3-dihydro-51-hydroxy-2-oxo CTX3C	51-hydroxy-2-oxoCTX3C	1054.6	/	/
2,3-dihydro-2,3,51-trihydroxy CTX3C	2,3,51-trihydroxyCTX3C	1072.6	/	/
A-seco-2,3-dihydro-51-hydrox yCTX3C	A-seco-51-hydroxyCTX3C	1058.6	/	/
M-seco-CTX3C	/	1040.6	/	/
M-seco-CTX3C methylacetal	/	1054.6	/	/
Caribbean ciguatoxin group (C-CTX1 and derivatives)				
Caribbean ciguatoxin-1	C-CTX1	1140.7, 1140.6	0.1	/
Caribbean ciguatoxin-2	C-CTX2	1140.7	0.3	/
C-CTX-analogues	10 additional analogues C-CTX3-12	1126.6, 1140.6, 1142.6, 1156.6, 1158.6	/	/

Indian ciguatoxin group (I-CTX1 and derivatives)				
Indian Ocean ciguatoxins 1-6	I-CTX1-6	1140.6, 1156.6, 1138.6, 1154.6	/	/
GAMBIERDISCUS METABOLITES OTHER THAN CTXs				
MTX	MT (maitotoxin), MTX, MTX-1	3422	/	BOC sciences, Alfa Chemistry, Wako
MTX2	MTX-2	3298 (sodium salt)	/	/
MTX3	MTX-3 (44-methyl gambierone)	1038.4858	/	Cifga
MTX4	MTX-4	3292.5	/	/
desulfo-MTX1	/	3299.6603	/	/
didehydrodemethyl-desulfo-MTX1	/	3283.6290	/	/
Gambieric acids A-D	GA-A	1056.6389		
	GA-B	1070.6546		
	GA-C	1187.7098		
	GA-D	1201.7254		
Gambierol	/	756.4451	/	/
Gambieroxide	/	1194.5648	/	/
Gambierone	/	1024.4704	/	Cifga

Action limit/toxicity

In the United States, the Food and Drug Administration (FDA) established consumer and industry guidance levels for ciguatoxins in finfish: 0.1 µg/kg Caribbean ciguatoxin-1 (C-CTX-1) equivalents and 0.01 µg/kg Pacific ciguatoxin-1 (P-CTX-1) equivalents. For Indian ciguatoxins, guidance levels have still to be established[6].
While there is no regulatory level in Europe for CTXs, the European Food Safety Authority (EFSA) adopted a concentration of 0.01 µg P-CTX-1 equivalents/kg fish, to cover all CTX-group toxins that could be present in fish[7].
For some toxins, Toxic Equivalence Factor have been proposed by EFSA or through the Eurociguatera project. However, TEF are not available for all identified toxins.

Analytical methods

Several analytical approaches have been developed and applied for CP analysis, including screening assays (in vitro assays, in vivo bioassays and immunoassays) and confirmatory methods ((U)HPLC-MS/MS or HRMS).
The mouse bioassay (MBA) has been widely used to detect CTX-group toxins in fish. However, according to both ethical reasons (animal welfare) and technical limitations (poor specificity and sensitivity), MBA was considered inappropriate for CP analysis.

In vitro (cytotoxicity and receptor binding) assays provide sufficient detection capability and can detect all active analogues of toxins. However, they do not provide information on analogue profiles.
Immunochemical methods, mostly enzyme-linked immunosorbent assays (ELISA), are fast and easy to apply. Antibodies used in ELISA kits are mostly specific to regional group of toxins and are not able to detect toxins from other regions. They do neither provide information on analogues profile nor allow reliable quantification.
Liquid chromatography-tandem mass spectrometry ((U)HPLC-MS/MS or HRMS) methods allow specific detection of individual analogues of P-, C- and I-CTX-group toxins and they would be of value for the quantification in fish extracts.

As toxin standards are scarce, a two-step approach is currently recommended[8] : first, using a screening method, followed by a further confirmation of the presence of toxin analogues with a confirmatory method such as LC MS/MS. The United States had defined advisory toxin levels for consideration as well as analytical approach to be applied in monitoring and control[9] . The system is based on a screening based on semi quantitative in vitro mouse neuroblastoma cell assay (N2A MTT) and later toxin confirmation by LC/MS MS[10]. A similar approach has been proposed in Australia[11] and also in Europe during the EuroCiguatera project[12 13].

It should be highlighted that, due to the low targeted concentrations of CTXs in fish, a quite elaborate sample preparation is required before LC-MS/MS, both to concentrate and to remove potential interfering compounds (e.g. lipids) from the sample. On the other hand, given to the large number of CTX analogues, availability of analytical standards represents one of the limitation for method development and validation. Currently, only CTX3C is commercially available as shown in Table 1.

To obtain reliable results for risk assessment and to achieve comparability between screening assays and LC MS/MS (e.g. N2A or RBA), two analytical aspects need to be addressed: toxicologically relevant analogues should be quantitatively analyzed and toxicity equivalency factors (TEFs) should be determined.

Presence in Europe

Until the 2000s, the occurrence of CP was thought to be limited to regions between latitudes 35°N and 35°S (i.e., the Pacific and Indian Oceans and Caribbean Sea). Detection of CP in non-endemic areas have ever since been shown in areas such as the Macaronesia (Canary Islands, Madeira, Azores, Cape Verde), the coast of Cameroon in West Africa, the Mediterranean (Balearic Islands, Cyprus, Crete) and the western Gulf of Mexico.

As mentioned in the EuroCiguatera project, France, Germany, Portugal and Spain have reported thirty-four ciguatera outbreaks from 2012 to 2019, including 209 cases. Outbreaks due to consumption of autochthonous fish have been reported by Spain and Portugal. The most frequent fish genus involved in the autochthonous outbreaks were Seriola and Epinephelus. In almost 60% of the autochthonous outbreaks, the fish was captured by sport fishing.

Germany and France reported outbreaks due to consumption of imported fish, with Lutjanus as the most frequent fish genus reported in imported outbreaks. In Germany, six outbreaks with 65 CFP cases were registered between 2012 and 2017. All patients suffered from neurologic and nearly 20% of the patients were hospitalized. All outbreak cases were caused by imported fish from India, Indonesia and Vietnam[14].

The occurrence in Europe, excluding tropical overseas territories, was very low (0.0054 cases per 100,000 inhabitants per year). However, the observed incidence rate was double in the Canary Islands (0.47 cases/100,000 inhabitants) compared to Florida (0.2 cases/100000 inhabitants observed from 2000 to 2011).

The EuroCiguatera project has also shown that toxicity of Gambierdiscus and Fukuyoa extracts from the Canary Islands and the Mediterranean Sea was mainly associated with MTXs, gambierone and gambieric acids, with no precursors of CTXs detected.

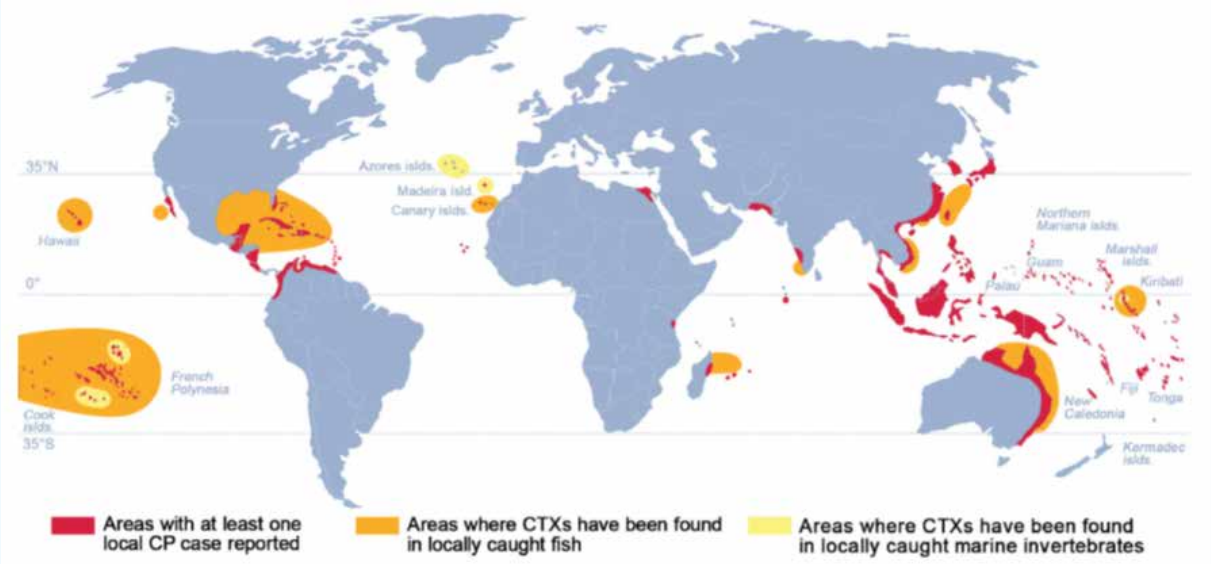


Figure 1 Illustration of the current global distribution of CP cases[15]

1 *2021, do Prado Leite et al. Experimental Evidence of Ciguatoxin Accumulation and Depuration in Carnivorous Lionfish. https://doi.org/10.3390/toxins13080564*
2 *2011, Rongo and van Woesik. Ciguatera poisoning in Rarotonga, southern Cook Islands. https://doi.org/10.1016/ j.hal.2010.11.005*
3 *2020, Estevez et al. Use of Mass Spectrometry to Determine the Diversity of Toxins Produced by Gambierdiscus and Fukuyoa Species from Balearic Islands and Crete (Mediterranean Sea) and the Canary Islands (Northeast Atlantic).doi:10.3390/toxins12050305*
4 *2021, FAO. Report of the expert meeting on ciguateria poisoning*
5 *Based on 2010, EFSA. Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group & and 2021, EFSA*
6 *2021, FDA. Fish and Fishery Products Hazards and Controls Guidance Fourth Edition – June 2021*
7 *2010, EFSA. Scientific Opinion on marine biotoxins in shellfish – Emerging toxins: Ciguatoxin group*
8 *2020, FAO. Report of the expert meeting on ciguateria poisoning*
9 *2021, FDA. Fish and Fishery Products Hazards and Controls Guidance Fourth Edition – June 2021*
10 *2009, Dickey and Plakas. Ciguatera: A public health perspective*
11 *2010, Stewart at al. Establishing a public health analytical service based on chemical methods for detecting and quantifying Pacific ciguatoxin in fish samples. https://doi.org/10.1016/ j.toxicon.2009.07.028*
12 *2021, EFSA. Risk characterisation of ciguatera poisoning in Europe*
13 *Caillaud, A., Eixarch, H., de la Iglesia, P., Rodriguez, M., Dominguez, L., Andree, K.B., et al., (2012). Towards the standardisation of the neuroblastoma (neuro-2a) cell-based assay for ciguatoxin-like toxicity detection in fish: application to fish caught in the Canary Islands. Food Addit. Contam.: Part A 29 (6),1000–1010. https://doi.org/10.1080/19440049.2012.660707*
14 *2019, Friedemann. Tropical fish poisonings in Germany 2012–2017 - What is ciguatera? https://doi.org/10.1016/j.ijid.2018.11.318*

Applications of droplet digital PCR (ddPCR) for food authenticity testing @ILVO

Taverniers, I, Derycke, S, Van Poucke, C, De Loose M (2022)

Instituut voor Landbouw-, Voedings- en Visserij- onderzoek (ILVO), Burg. Van Gansberghelaan 115, B-9820 Merelbeke, BELGIE. Contact: isabel.taverniers@ilvo.vlaanderen.be

Abstract

Droplet digital PCR is a very valuable tool for authenticity analyses of food products. The more traditional techniques for species determination in fish, DNA sequencing and high resolution melting analysis (HRMA), are less applicable if several fishery species are present in mixed products, and also do not allow quantitative analysis. Semi-quantification of Atlantic salmon and the detection of fraud based on replacement of the salmon by other, cheaper species, proved to be possible in mixtures based on droplet digital PCR (ddPCR). Optimizing, validating and implementing ddPCR methods for food allergens are also the subject of this article. Specific for soybean ddPCR determination in complex and processed food, such as e.g. baked cookies, a specificity test as well as 2-part sensitivity test as part of ddPCR validation are described. Sensitive detection of soy via ddPCR appears to be possible down to approx. 10 ppm soy protein in a cookie matrix containing multiple allergens.

Introduction: food authenticity and PCR technology

The label on the packaging of a food product contains information about the used ingredients, the applied method of preparation, any allergens present, nutritional value. However, infringements can occur on the composition, origin or production method of a raw material or food product, as a result of which the label in such cases does not correspond to reality. Examples of product authenticity infringements include: the use of a dilution of or a cheaper alternative for a particular ingredient, misidentification of the used technology, geographic origin, generic name. Specific and sensitive analytical methods are required to detect such violations of authenticity. ILVO has an extensive range of chemical, biochemical, enzymatic, immunological and molecular methods to detect and identify species and varieties. Rapid identification techniques that use species-specific primers to amplify specific targets include real-time qPCR, digital PCR (dPCR) and highresolution melting curve analysis (HRMA).

After DNA extraction from a sample, the amplification of specific genes or DNA sequences that are characteristic of e.g. allergens or specific target species, is performed via PCR. Fluorescent probes are used during multiplication, whereby the emitted light signal is directly proportional to the number of copies of the target gene. The detection of the fluorescent signal takes place in real time, such that the result can be read immediately on the computer. In digital PCR, the amplification does not take place in one reaction mix, but in thousands to tens of thousands of individual compartments, i.e. droplets in droplet digital PCR (ddPCR) or chambers in chamber digital PCR (cdPCR). The number of compartments that show a positive signal is counted per analysis. The final result, absolute quantification, is obtained on the basis of a statistical calculation.

The advantages of digital PCR (dPCR) over conventional real-time PCR (qPCR) include accurate absolute quantification of a target sequence or gene without the need for standards or calibrators and the much lower impact of inhibitory components present in the PCR reaction and originating from the sample matrix. Both chamber digital PCR (cdPCR) and droplet digital PCR (ddPCR) are widely used in medical and clinical, plant disease and nutritional diagnostics, to name just a few application domains. Digital PCR offers many possibilities for specific and highly sensitive detection and quantification, in a fast and flexible way.

Within ILVO, ddPCR is used as an innovative technology for, among other things, the identification of Atlantic salmon in mixed fishery products (Deconinck et al. 2021); detection and quantification of a number of commercially important fishery species (plaice, sole, flat and hollow oysters) in the North Sea on the basis of free DNA molecules in the seawater (so-called eDNA or "environmental DNA"); species-specific determination of species in eDNA (environmental DNA) samples from Flemish rivers; determination of construct copy numbers after genetic transformation of plants via Agrobacterium rhizogenes; comparison between qPCR and ddPCR for quantification of STEC (Escherichia coli) in bovine faeces; and finally detection of food allergens (species detection) in food. The first and last application are the subject of this article and will therefore be explained in more detail.

Quantification of fishery species

Fish is an important part of our diet. Due to the growing population, the pressure on our fish populations is increasing and this may jeopardize the supply and availability of a number of fish species. In some cases this is accompanied by fraudulent practices whereby fish species are replaced by other – often cheaper – species. Processed products often contain only the muscle tissue of the fish, so that the external characteristics to recognize the fish are no longer present. These processed fish products can be identified via DNA barcoding, in which a piece of DNA is sequenced and the sequence obtained is compared with a reference database.

IWithin the international Seafoodtomorrow project (https://seafoodtomorrow.eu/), ILVO has drawn up a reliable reference database for the fish species traded on the European market, and has shown through a targeted "DNA barcoding" study in processed fish products that cod (Gadus morhua) and especially sole (Solea solea) on the Belgian market are sometimes replaced by cheaper species, and that this substitution can occur at all levels of the food processing chain (Deconinck et al. 2020).

The Seafoodtomorrow project further focuses on the development of innovative methods to guarantee the quality and safety of fishery products to the consumer. For example, we are now able to analyze eight different salmon species using qPCR and high resolution melting analysis (HRMA) in processed products (Figure 1) Atlantic salmon (Salmo salar) represents more than 50% of all salmon sold to consumers worldwide is offered (FAO, 2020). Atlantic salmon is very similar in taste, appearance and texture to the Pacific salmon species but is less expensive because the species is farmed in aquaculture. Pacific salmon is therefore often (17%) replaced by Atlantic salmon (Luque and Donlan, 2019), while Atlantic salmon is sporadically replaced by the cheaper char and rainbow trout. The major advantage of the newly developed HRMA method is that salmon processing has no effect on detection (with the exception of canning) and that the result of the analysis is available within four hours (Monteiro et al, 2021). The HRMA method was then applied to 81 salmon samples from Belgium, Poland and Portugal and showed that only one sample was fraudulent: a wild salmon sample of Onchorhynchus nerka was replaced by the cheaper aquaculture species Salmo salar (Monteiro et al. 2021).

Figure 1 Identification of salmon samples via HRMA and qPCR. A piece of salmon fillet from one species is used to perform DNA extraction, then the DNA is added to a qPCR mix where primers are added so that only a piece of mitochondrial DNA from salmon species is multiplied. The DNA sequence of this piece is different for each salmon species, resulting in a specific melting curve.

However, "DNA barcoding" and HRMA also have their limitations: they cannot be used for products where more than one type is present (such as surimi, fish soup, pâté…) and quantification of the amount of a certain type in the product is also not possible. . This is where ddPCR can provide added value: when multiple species are present in a product, primers and probes can be developed that only detect the DNA of the target species and at the same time determine the amount of DNA of that target species in the sample. The assumption here is that the amount of DNA extracted from the sample correlates with the amount of fish in the fish product. To test this, a ddPCR assay was developed and validated for Atlantic salmon (Salmo salar) within the framework of the Seafoodtomorrow project within ILVO. The developed ddPCR assay only detects the DNA of Atlantic salmon and targeted lab tests with mixed samples of two salmon (Salmo salar and Oncorhynchus mykiss) containing different weight percentages of Atlantic salmon show a strong linear relationship between the amount of Atlantic salmon in the sample and the number DNA copies measured (Figure 2).

Figure 2 Identification of mixed salmon samples via ddPCR. A sample that can contain several salmon species is used to perform DNA extraction, then the DNA is added to a ddPCR mix in which primers and a probe are added that only multiply a piece of mitochondrial DNA from Atlantic salmon (Salmo salar). In test samples in which tissue from S. salar and Oncorhynchys mykiss were mixed in different proportions, the number of copies of DNA from the ddPCR was found to be correlated with the weight percentage of Salmo salar tissue added to the mixture.

As the fish products are prepared (e.g. by cooking, freezing, smoking, gravad lax) the DNA can be broken down and therefore have an impact on the number of DNA copies that are detected. Freezing and canning the salmon results in a lower DNA copy number, making it important to observe the preparation method for correct quantification. Finally, the ddPCR assay was tested on 30 Atlantic salmon and 16 Pacific salmon samples from the supermarket. Not a single sample turned out to be fraudulent, and the amount of Atlantic salmon in complex meals could be determined semi-quantitatively when information about the preparation of the meal and about the other ingredients is available (Deconinck et al. 2021). These results are an important step to check the labeling of mixed fish products for correct naming and to make a rough estimate of the stated percentages of the fish in the meal.

Food allergen detection

European Regulation 1169/2011 requires the labeling of 14 food groups that may be potentially allergenic if used as an ingredient. In addition to this mandatory labeling of food products, there is precautionary allergen labeling (PAL) for labeling traces of allergenic products or species. This "may contain traces of" labeling is not mandatory but strongly recommended in case there is an indication - based on risk assessment - that the allergen could be present in the final product. The main allergens of animal origin are milk, egg, fish, molluscs and crustaceans. Gluten, soy, peanut, tree nuts, celery, sesame, mustard and lupins are the best known and characterized plant allergens. Monitoring the potential presence of traces of allergenic components is based, among other things, on tracing, supplemented by analytical tests during and at the end of production. Worldwide and within the EU there are no recommendations regarding the appropriate technology or methodology for allergen determination. However, there is consensus within the EU about the "unit" to be used for the quantification of allergens, i.e. mg of total protein of the allergenic ingredient per kg of product. Allergens can be detected using various technologies, each of which has its own specific pros and cons.

The DNA-based PCR technique is universal, extremely stable and sensitive, but has no direct link with an amount (mg) of allergenic protein. Protein-based technologies such as enzyme-linked immunosorbent assay (ELISA) or lateral flow dipstick (LFD) tests seem to have this at first. However, the target used in ELISA is often one or a group of very specific epitope(s) of one protein or several proteins that are specific for the ingredient to be detected. From an analytical point of view, the total amount of protein of the allergenic ingredient per kg of product is also not determined in this case, but the number of epitopes of a specific protein of the ingredient is. The epitopes that are detected are not necessarily those involved in the allergic reaction. In other words, conversion to the total amount (mg) of allergenic protein is also necessary for protein-based techniques.

Finally, protein detection by means of chemical analysis techniques, such as e.g. liquid chromatography (liquic chromatography, LC) coupled to tandem mass spectrometry (MS/MS), like all protein-based technologies, is highly dependent on food processing such as e.g. thermal treatments. In addition, chemical analyzes often require high-tech and expensive equipment and related training of the operators.

Within the EFSA project ThrALL (Detection and quantification of allergens in food and minimum eliciting doses in food allergic individuals, GP/EFSA/AFSCO/2017/03, 2017-2022) one of the objectives was to test ddPCR for the detection of trace amounts of soy, peanut, hazelnut and almond in complex and highly processed matrices such as broth and chocolate.

Figure 3 shows the analytical flow followed in development and implementation of ddPCR assays. Testing and optimizing ddPCR methods is preceded by possible optimization and in-house validation of a basic qPCR assay. Existing qPCR primers/probes (see Fig. 3, middle) were tested in ddPCR on pure soybean, peanut, hazelnut and almond respectively.

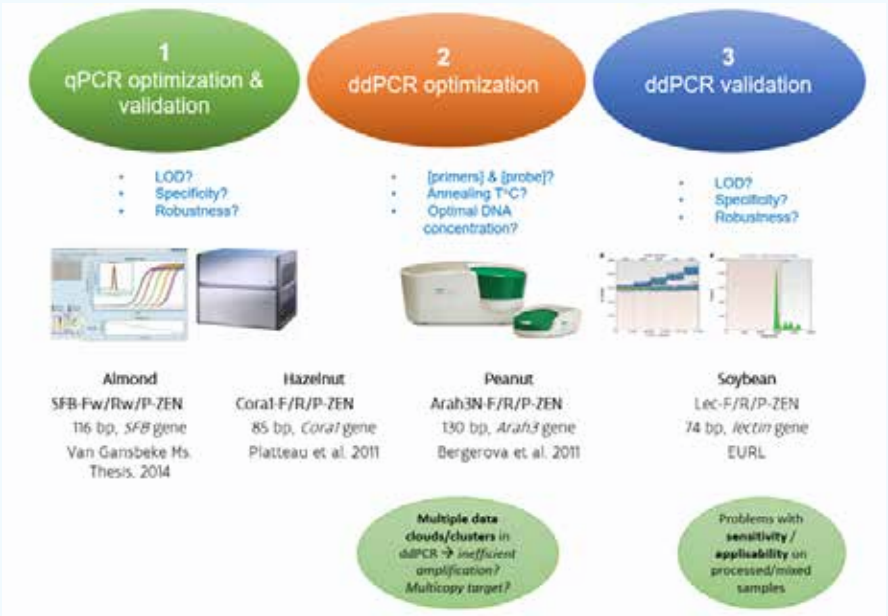


Figure 3 Flow from and main criteria within qPCR optimization and validation (1), to ddPCR optimization (2) and ddPCR validation (3) with the aim of converting an existing qPCR assay to a ddPCR assay. In the middle, the primers/probes used are listed per target allergen, as well as the status in the flow at the end of the project. At the bottom in green are some typical issues that can classically occur with ddPCR at that step in the flow.

On DNA extracted from pure raw materials, i.e. ground powder of soybeans, peanuts, hazelnuts and almond nuts, within the ddPCR assay validation a theoretical LOD determination is first performed in 2-fold on a dilution series from 500 to 0.5 pg/µl pure soy DNA (CRM). This dilution series is first performed in duplicate, after which the lowest detectable concentrations (16 – 7.8 – 3.9 – 2 pg/µl pure soybean DNA) are run again in 8 replicates each time. The lowest concentration that then gives at least 3 positive droplets for all 8 replicates is defined as the absolute LOD, here determined at 7.8 pg/µl pure soy DNA or 7 cp/µl soy-specific target DNA (Le1). An example of specificity determination of the soybean ddPCR specificity test is shown in Figure 4.

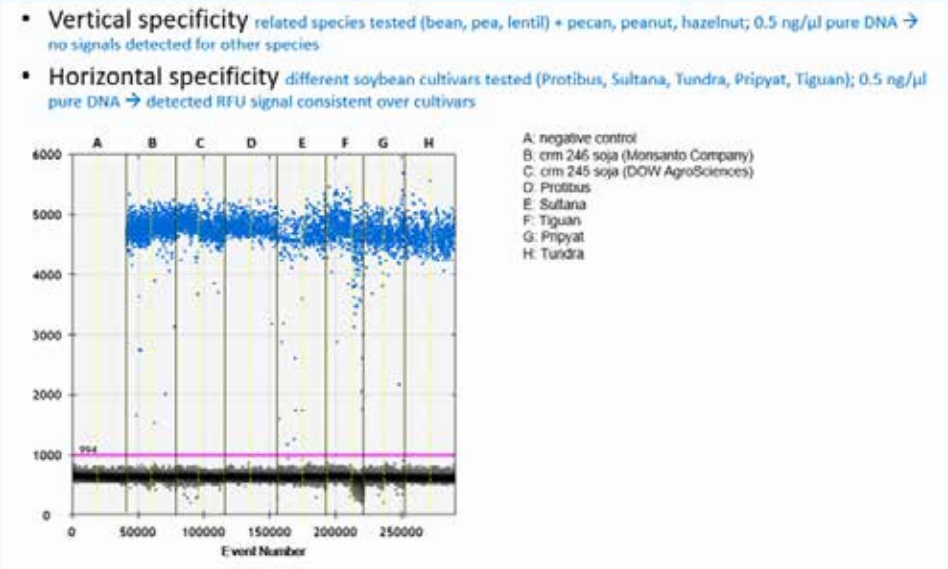


Figure 4 Illustration of the soy ddPCR specificity assessment in 2 parts

Finally, a practical and relative LOD determination and applicability tests are performed on lower concentrations of a mixture of allergens in a processed and mixed food product. Baked cookies are a typical example of such a matrix, used within the EFSA-ThrALL project (Fig. 5). Seven different allergens were added to this matrix, including soy, peanut, hazelnut and almond. An illustration of the relative and practical LOD determination for soy in this cookie material is shown in Fig. 6.

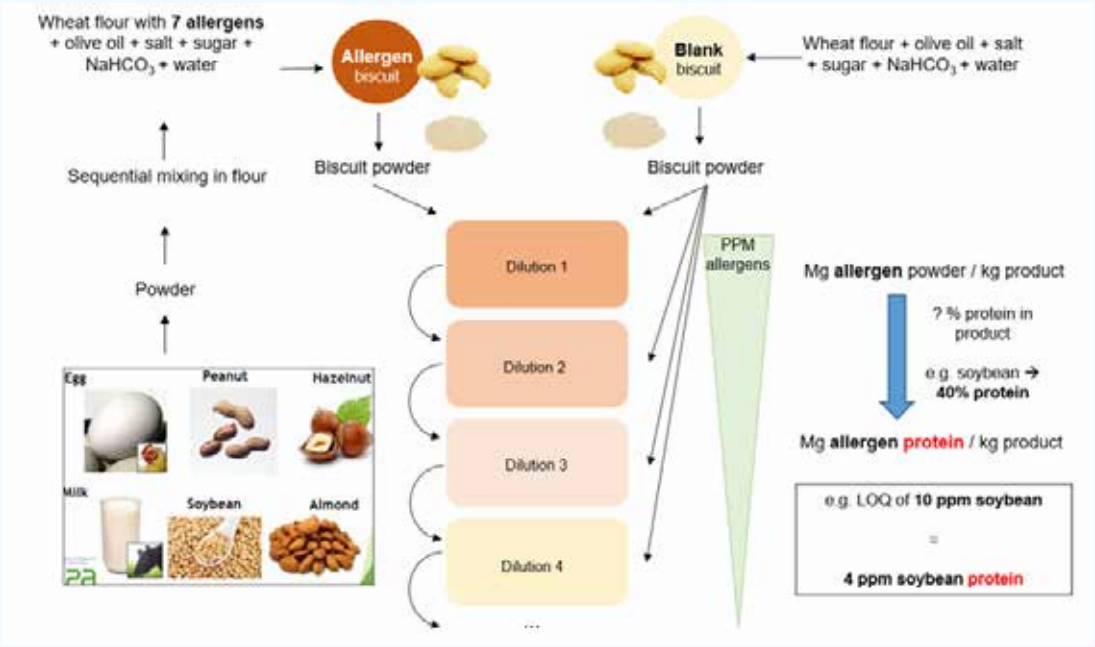
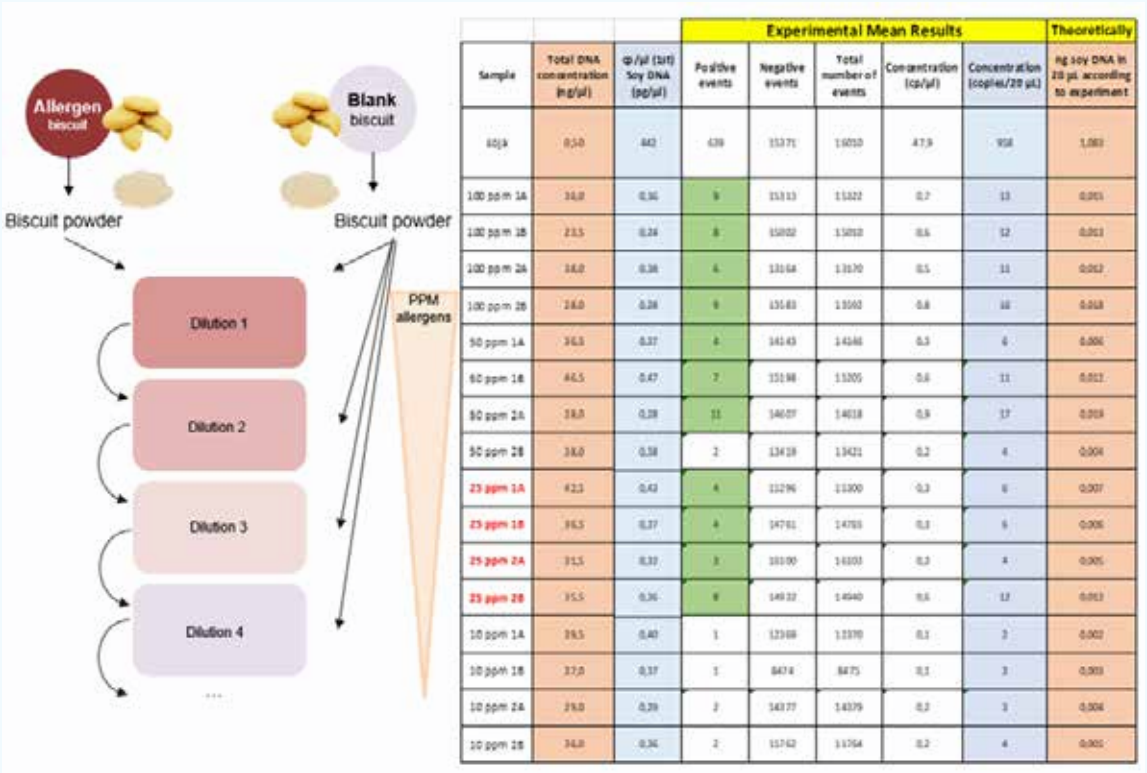


Figure 5 Production process of in-house allergens-containing cookies



			Experimental Mean Results					Theoretically
Sample	Total DNA concentration (ng/µl)	cp/µl (1st) Soy DNA (cp/µl)	Positive events	Negative events	Total number of events	Concentration (cp/µl)	Concentration (copies/20 µL)	ng soy DNA in 20 µL according to experiment
60/a	0,50	842	639	15371	16010	47,9	958	1,083
100 ppm 1A	34,0	0,36	9	15313	15322	0,7	13	0,001
100 ppm 1B	23,5	0,24	8	15002	15010	0,6	12	0,001
100 ppm 2A	34,0	0,36	6	13164	13170	0,5	11	0,002
100 ppm 2B	24,0	0,26	9	15183	15192	0,6	12	0,018
50 ppm 1A	16,3	0,17	6	14143	14148	0,3	6	0,004
50 ppm 1B	44,5	0,47	7	15198	15205	0,6	11	0,011
50 ppm 2A	28,0	0,28	11	14007	14018	0,9	17	0,010
50 ppm 2B	24,0	0,58	2	13419	13421	0,2	4	0,004
23 ppm 1A	42,3	0,43	4	11296	11300	0,3	6	0,007
23 ppm 1B	26,5	0,27	4	14761	14765	0,3	5	0,005
25 ppm 2A	31,5	0,33	3	10100	10103	0,3	4	0,005
25 ppm 2B	35,5	0,36	9	14932	14940	0,6	12	0,013
10 ppm 1A	39,5	0,40	1	12368	12370	0,1	3	0,002
10 ppm 1B	37,0	0,37	1	8474	8475	0,1	3	0,003
10 ppm 2A	29,0	0,29	2	14377	14379	0,2	3	0,004
10 ppm 2B	34,0	0,34	2	11762	11764	0,2	4	0,005

Figure 6 Relative and practical LOD determination for soybean ddPCR in cookie mix. The different concentrations of soy allergen in the matrix, visible in the 1st column in the table, are tested in 2 separate DNA extracts (1 and 2) and each extract in duplicate (A and B). The practical LOD is defined as the lowest concentration (in ppm total allergen in the matrix) at which min. 3 positive droplets were still measured over the 4 replicates – here 25 ppm soy, at ~40% protein content for pure soybeans, this corresponds to ~ 10 ppm soy protein as practical LOD in biscuits.

Conclusions

Both qPCR and ddPCR have important applications in authenticity analyses of food products. Both methods allow for rapid (within 5 hours) identification of the ingredients in processed food. For fishery products, only identification of canned fish products is not possible, presumably because the DNA has broken down too much. To check whether the percentage of a certain fish species stated on the label is correct, it was investigated whether quantification of the number of copies of a gene can be used as a proxy for this. Reliable quantification is possible for mixed samples containing only fish tissue. The translation to more complex multi-ingredient food products can only be done semi-quantitatively due to the presence of ingredients that do not contain DNA. When it comes to allergen analysis, ddPCR can detect very low concentrations: in the case of soybean, the absolute limit of detection is 7.8 pg/µl DNA or 7 copies/µl DNA, while the relative limit of detection was determined to be 25 ppm of soy in cookies or, taking into account 40% protein content for soybeans, 10 ppm total soy protein in cookies.

Referentces

Luque, G.M., and Donlan, C.J. (2019). The characterization of seafood mislabeling: A global meta-analysis. Biological Conservation 236, 556-570.

Deconinck D., Hostens K., Taverniers I., Volckaert F.A.M., Robbens J., and Derycke S. (2021). Identification and semi-quantification of Atlantic salmon in processed and mixed seafood products using Droplet Digital PCR (ddPCR). Food and Chemical Toxicology, 154 112329, 11 pages. https://doi.org/10.1016/j.fct.2021.112329

Monteiro, C. S., Deconinck, D., Eljasik, P., Sobczak, M., Derycke, S., Panicz, R., Kane, N., Mazloomrezaei, M., Devlin, R. H., & Faria, M. A. (2021). A fast HRMA tool to authenticate eight salmonid species in commercial food products. Food and Chemical Toxicology, 156. https://doi.org/10.1016/j.fct.2021.112440

Platteau C, De Loose M, De Meulenaer B, Taverniers I. (2011). Detection of Allergenic Ingredients Using Real-Time PCR: A Case Study on Hazelnut (Corylus avellena) and Soy (Glycine max). Journal of Agricultural and Food Chemistry, 59(20):10803-14. https://doi.org/10.1021/jf202110f

Bergerová E, Brežná B, Kuchta T. (2011). A novel method with improved sensitivity for the detection of peanuts based upon single-tube nested real-time polymerase chain reaction. European Food Research and Technology, 232(6):1087-91. https://doi.org/10.1007/s00217-011-1484-y

Van Gansbeke B. (2014). An authenticity test for marzipan: SYBR Green I and TaqMan real-time PCR assays for almond (Prunus dulcis) and apricot (Prunus armeniaca) for detection of traces of persipan. Ms thesis, Ms of Science, Promotors G. Beny, A. Dutta, I. Taverniers. 54 pp.

The impact of food processing on food allergens: allergenic reactions and detection

Christof Van Poucke[1], Maxime Gavage[2], Kaatje Van Vlierberghe[1], Isabel Taverniers[1], Marc De Loose[1] and Nathalie Gillard[2]

(1) Flanders Research Institute for Agriculture, Fisheries and Food, Technology and Food Science Unit, Brusselsesteenweg 370, BE-9090 Melle, Belgium
(2) CER Groupe, Rue du Point du Jour 8, 6900, Marloie, Belgium

Food allergens

When a food allergen-sensitive individual is exposed to the allergenic food, an adverse health effect caused by an immune response occurs (1). For the majority of the population this food product is harmless, but these food allergen-sensitive individuals show an abnormal immune-mediated response (2). The major differences between food allergy and food sensitivities (or food intolerances) is the involvement of the patient's immune system (3) and the fact that, in food allergy, proteins are the elicitor in the majority of the cases, while with a food intolerance any food component can be involved e.g. a sugar (lactose) in lactose-intolerance (4). Within food allergy a division can be made between IgE-mediated food allergies, giving a rapid and reproducible effect after consumption of the food product, and non-IgE-mediated reactions which generally show a delayed and less severe effects (5).

Food allergies can give rise to diverse symptoms, however within the scientific community only those which occur during standardised challenge tests such as a double-blind placebo-controlled oral food challenge (DBPCFC) in IgE-mediated sensitised patients are recognized to be caused by food allergies (6). A food allergy can give rise to reactions all over the body (skin, respiratory tract, gastrointestinal-system, vascular system), and the symptoms can vary from mild reactions (hives, vomiting, …) to life threatening situations (anaphylactic shock) (Figure 1).

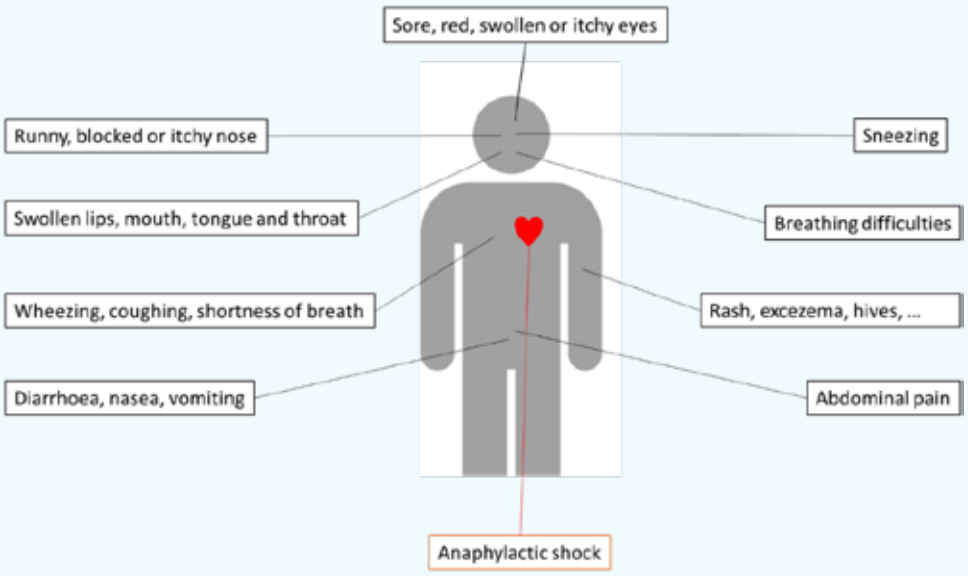


Figure 1: Overview of possible IgE-mediated reactions after ingestion of a food allergen by a sensitised patient.

Until recently, no cure to treat food allergies was recognized and consequently a food allergic patient could only protect him/herself from adverse reactions by avoidance of the relevant food product (7). Recently in the US the FDA approved the first drug for treatment of peanut allergy for children (https://www.fda.gov/news-events/press-announcements/fda-approves-first-drug-treatment-peanut-allergy-children). In Europe no such treatment has been recognized so far, and the European legislation (Regulation 1169/2011/CE) has been put in place in order to help the allergic patient to avoid consuming these allergens. This regulation obligates the labelling of 14 potentially allergenic food products (Table 1) when used as an ingredient in food products.

Table 1 - List of allergenic ingredients and substances causing intolerances that require labelling and permitted exemptions from Annex II of Consumer Information Regulation (EC) 1169/2011	
1.	Cereals containing gluten, namely: wheat, rye, barley, oats, spelt, kamut or their hybridised strains, and products thereof, except: (a) wheat based glucose syrups including dextrose[1] (b) wheat based maltodextrins[1] (c) glucose syrups based on barley (d) cereals used for making alcoholic distillates including ethyl alcohol of agricultural origin
2.	Crustaceans and products thereof
3.	Eggs and products thereof
4.	Fish and products thereof, except: (a) fish gelatine used as carrier for vitamin or carotenoid preparations (b) fish gelatine or Isinglass used as fining agent in beer and wine
5.	Peanuts and products thereof
6.	Soybeans and products thereof, except: (a) fully refined soybean oil and fat1 (b) natural mixed tocopherols (E306), natural D-alpha tocopherol, natural D-alpha tocopherol acetate, and natural D-alpha tocopherol succinate from soybean sources (c) vegetable oils derived phytosterols and phytosterol esters from soybean sources (d) plant stanol ester produced from vegetable oil sterols from soybean sources
7.	Milk and products thereof (including lactose), except: (a) whey used for making alcoholic distillates including ethyl alcohol of agricultural origin (b) lactitol
8.	Nuts, namely: almonds (*Amygdalus communis L.*), hazelnuts (*Corylus avellana*), walnuts (*Juglans regia*), cashews (*Anacardium occidentale*), pecan nuts (*Carya illinoinensis (Wangenh.)* K. Koch), Brazil nuts (*Bertholletia excelsa*), pistachio nuts (*Pistacia vera*), macadamia or Queensland nuts (*Macadamia ternifolia*), and products thereof, except: (a) nuts used for making alcoholic distillates including ethyl alcohol of agricultural origin
9.	Celery and products thereof
10.	Mustard and products thereof
11.	Sesame seeds and products thereof
12.	Sulphur dioxide and sulphites at concentrations of more than 10 mg/kg or 10 mg/L in terms of the total SO_2 which are to be calculated for products as proposed ready for consumption or as reconstituted according to the instructions of the manufacturers
13.	Lupine and products thereof
14.	Molluscs and products thereof

[1]And the products thereof, in so far as the process that they have undergone is not likely to increase the level of allergenicity assessed by the Authority for the relevant product from which they originated.

Food processing

Most of the food products we consume have been processed, either by industry or at home during cooking. In supermarkets, the share of industrial processed foods is still rising. This is mainly caused by the increase in global population, convenience (lack of time, effort), seasonal availability,… (8). Where thermal processing, one of the most frequently applied food processing techniques, is mainly used to prolong preservation (sterilization/pasteurization) of food products, other reasons for industrial food processing are the positive impact on food quality (taste, flavour and texture), obtaining useful by-products or better marketability (9). Often a complex combination of different food processing techniques is applied, in some cases leading to ultra-processed foods. The applied processing techniques are mostly not mentioned on the label of the food package.

The impact of food processing on allergenicity

In order to have an IgE-mediated reaction to a food allergen, the epitope(s) of the protein need(s) to be recognized by the IgE antibody (10). Consequently, alterations to the protein, and more specific to the epitope(s), can have an effect of the allergenic potential of the food product. Food processing can induce such alterations to proteins leading to epitope(s) modifications resulting in both increased or decreased allergenicity (11). This phenomenon can explain why for certain foods there is a difference in allergenicity between processed and non-processed products. Conformational epitopes might for instance be lost due to heating and the associated denaturation of proteins, while with linear epitopes increased allergenicity might occur because certain regions of the protein become more accessible for the antibodies to bind to (neoallergens) (Figure 2).
One example that nicely demonstrates the effect of processing is the case of peanuts. Where peanut is one of the most

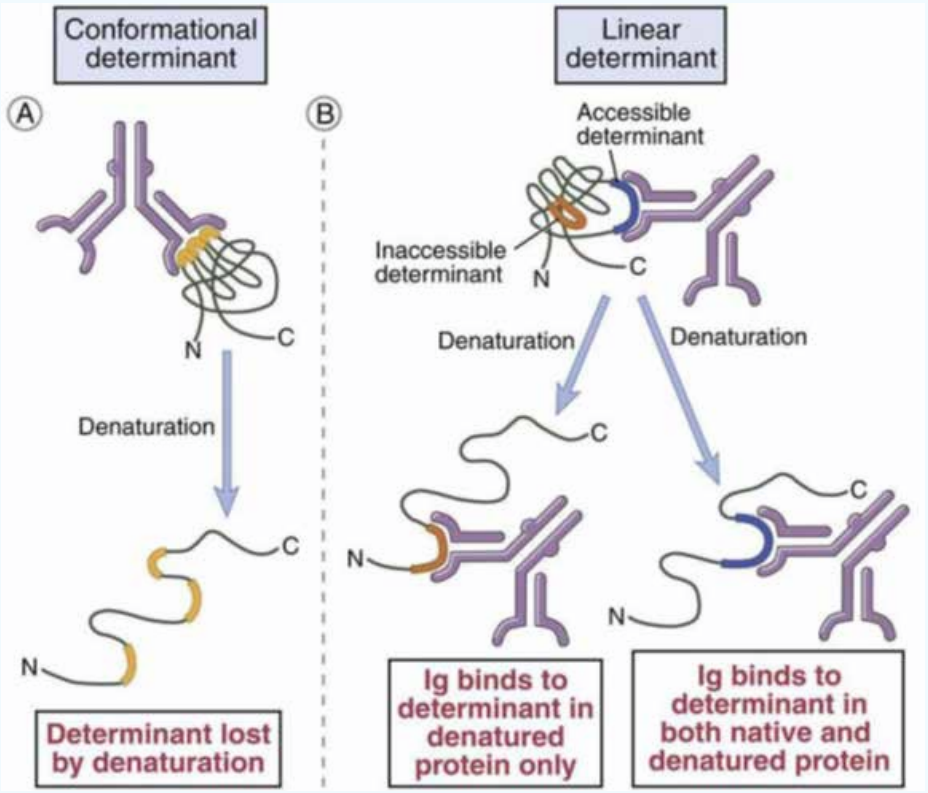


Figure 2: Possible effects of food processing on epitopes (12).

prevalent and severe food allergies in Western countries (13) the adverse is the case in Asia (14). This phenomenon cannot be explained by consumption rates as these are similar, neither by genetic differences in population as the prevalence of peanut allergy in the Asian population living in Western countries is comparable to the prevalence in the Western population (15). However, it is correlated to dietary habits where in Asian countries mainly boiled peanuts are consumed, while in our Western diet peanuts are mainly roasted. These two different food processing techniques have a different impact on the structure of the proteins, and consequently the epitopes, resulting in a different allergenicity (16).

The opposite effect of food processing can be illustrated by the Pollen Food Allergy Syndrome (PFAS) or Oral Allergy Syndrome (OAS) (17). In this case, the food allergy is actually a secondary food allergy in which antibodies for e.g. birch pollen show cross-reaction towards proteins occurring in hazelnuts and/or apples (18, 19). These proteins are often more labile, resulting in both less severe and more local reactions than might be the case with other allergens (20), but also make them more susceptible towards denaturation during heat treatment. As such, patients with an OAS will react when eating a raw apple or raw hazelnuts but will be able to tolerate e.g. applesauce or hazelnut paste (21, 22).

Detection of food allergens

According to the applicable legislation (Regulation 1169/2011/CE), it concerns the allergenic ingredient that needs to be detected in the food, not the eliciting factor (in casu the protein). Consequently, current applied methods in food allergen detection target different allergenic ingredient specific markers such as DNA, proteins or peptides instead of the eliciting epitope (3). When targeting DNA, PCR techniques are used to amplify the allergen thus species specific DNA (23), while for protein-based methods, antibodies are often used in ELISA's to detect the allergenic ingredient (24).

Both PCR and ELISA are well established techniques in food allergen detection and which, because of their relative low cost price and ease of operation, can both be performed in a lab environment as in industry. A major drawback for both techniques is however their susceptibility towards food processing (11). Although generally DNA is considered to be stable against many processing techniques, in some cases it may degrade, aggregate and/or become difficult to be extracted, which can result in false negative results (23). Therefore, it is important during method development to choose a stable species-specific target. Also the fact that PCR has no direct link to the eliciting factor (proteins) is often mentioned as drawback for this technique, although none of the used detection techniques necessarily have a link with the allergenic epitope (3). As described above, food processing might alter the structure of the proteins, making them no longer recognisable for the antibodies used in ELISA's, again impacting the outcome of the analysis (25). Furthermore, also the extraction of proteins might be influenced by food processing. Another drawback of ELISA's, just as PCR, is the fact that it can only target one analyte at the time, making the use of multiple tests necessary to cover a range of food allergens, resulting in an increased cost price. Finally, ELISA's might also suffer from cross-reactivity.
A technique that is becoming more and more widespread and which might overcome many of the above mentioned drawbacks is liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS). With LC-MS/MS the ingredient specific marker are peptides (26) obtained after extracting the proteins from the matrix and digesting these proteins with a specific enzyme (e.g. trypsin). An important step when using this technique is the selection of the target peptides, these do not only need to specific for the allergic ingredient (proteotypic peptides) but preferably are also chosen in function of stability during food processing (e.g. excluding modifications or reduced extractability due to processing) (27). Furthermore, LC-MS/MS allows multiplexing (combining multiple allergens in one detection method) which results in both gaining throughput time and reducing costs (28).

Although LC-MS/MS can solve many of the issues in food allergen detection, the absolute quantification of the allergen remains for all three techniques a point of attention where calibrators and conversion factors need to be chosen carefully (29).

Allersens project

The Belgian Federal Public Service of Health, Food Chain Safety and Environment financed the RT15/10 project ALLERSENS in which CER Groupe, ILVO, UNamur en UGent/VIB teamed up to develop such a processing robust LC-MS/MS method for food allergen detection focussing on milk, egg, peanut and hazelnut as food allergens.
In a first step, test materials of the four specific allergens were produced in the Food Pilot at ILVO. These materials only contained the target allergen and were submitted to often used processing techniques in the food industry such as dry heating, low pH, high fat content, … and also different geographical origin of the allergens was taken into consideration. Using these test materials processing stable proteotypic marker peptides were determined using a discovery proteomics approach (30-33).

To overcome known issues in quantification of food allergens, an isotope dilution strategy using a stable isotope-labelled concatemer which could be used as internal standard was developed. Such a concatemer is an artificial protein, recombinantly produced and constructed from isotope labelled biomarkers from the different proteins. When adding this internal standard at the first step of the sample preparation, the concatemer is submitted to enzymatic digestion together with all other proteins present in the extract resulting in stable isotope-labelled peptides. This approach, which had never been applied before in food allergen analysis, allows to overcome known limitations when using synthetic peptides, is cheaper than using isotope-labelled proteins and allows multiplexing (34).

In a next step of the project the identified marker peptides, and the developed quantification strategy using the isotope-labelled concatemer were combined into a targeted LC-MS/MS method allowing the simultaneous detection of hazelnut, peanut, milk and egg in cookie and chocolate. For the development and validation of this method a second type of test material was developed containing trace amounts of the four allergens in cookie and chocolate matrices. Prior to validation, the homogeneity of these matrices was evaluated in order to allow proper validation. Results of this validation study proved the suitability of the developed method to detect and quantify the four target allergens at the VITAL action limits (27). In a final step an inter-lab study was set up to prove the robustness of the method.

References

1. Berin MC, Sampson HA. Mucosal Immunology of Food Allergy. *Curr Biol* 2013;**23**:R389–R400.
2. Nowak-Wegrzyn A, Sampson HA. Adverse Reactions to Foods. *Medical Clinics of North America* 2006;**90**:97–127.
3. Poms RE, Klein CL, Anklam E. Methods for allergen analysis in food: a review. *Food Additives & Contaminants* 2004;**21**:1–31.
4. Tuck CJ, Biesiekierski JR, Schmid-Grendelmeier P, Pohl D. Food Intolerances. Nutrients 2019;**11**. doi:10.3390/nu11071684
5. Food Intolerance versus Food Allergy. 2017;**1**:3.
6. Ortolani C C, Bruijnzeel-koomen C, Bengtsson U, Bindslev-jensen C, Björkstén B, Høst A et al. Controversial aspects of adverse reactions to food: Adverse reactions to food. *Allergy* 1999;**54**:27–45.
7. Popping, B. & Diaz-Amigo, C. European Regulations for Labeling Requirements for Food Allergens and Substances Causing Intolerances: History and Future. J. AOAC Int 2018; **101**: 2-7
8. Sathe SK, Sharma GM. Effects of food processing on food allergens. *Molecular Nutrition & Food Research* 2009;**53**:970–978.
9. Verhoeckx KCM, Vissers YM, Baumert JL, Faludi R, Feys M, Flanagan S et al. Food processing and allergenicity. *Food and Chemical Toxicology* 2015;**80**:223–240.
10. Waserman S, Bégin P, Watson W. IgE-mediated food allergy. *Allergy, Asthma & Clinical Immunology* 2018;**14**:55.
11. Sathe SK, Teuber SS, Roux KH. Effects of food processing on the stability of food allergens. *Biotechnology Advances* 2005;**23**:423–429.
12. Cellular and Molecular Immunology - 6th Edition. https://www.elsevier.com/books/cellular-and-molecular-immunology/abbas/978-1-4160-3122-2 (accessed 9 Jun2020).

13. Sicherer SH, Sampson HA. Peanut allergy: emerging concepts and approaches for an apparent epidemic. J *Allergy Clin Immunol* 2007;**120**:491–503; quiz 504–505.
14. Lee AJ, Thalayasingam M, Lee BW. Food allergy in Asia: how does it compare? *Asia Pac Allergy* 2013;**3**:3–14.
15. Beyer K, Morrow E, Li XM, Bardina L, Bannon GA, Burks AW et al. Effects of cooking methods on peanut allergenicity. *J Allergy Clin Immunol* 2001;**107**:1077–1081.
16. Zhang T, Shi Y, Zhao Y, Tang G, Niu B, Chen Q. Boiling and roasting treatment affecting the peanut allergenicity. *Ann Transl Med* 2018;**6**. doi:10.21037/atm.2018.05.08
17. Mastrorilli C, Cardinale F, Giannetti A, Caffarelli C. Pollen-Food Allergy Syndrome: A not so Rare Disease in Childhood. *Medicina* 2019;**55**. doi:10.3390/medicina55100641
18. Mauro M, Russello M, Incorvaia C, Gazzola G, Frati F, Moingeon P et al. Birch-apple syndrome treated with birch pollen immunotherapy. *Int Arch Allergy Immunol* 2011;**156**:416–422.
19. Muluk NB, Cingi C. Oral allergy syndrome. *Am J Rhinol Allergy* 2018;**32**:27–30.
20. Werfel T, Asero R, Ballmer-Weber BK, Beyer K, Enrique E, Knulst AC et al. Position paper of the EAACI: food allergy due to immunological cross-reactions with common inhalant allergens. *Allergy* 2015;**70**:1079–1090.
21. Yu W, Freeland DMH, Nadeau KC. Food allergy: immune mechanisms, diagnosis and immunotherapy. *Nature Reviews Immunology* 2016;**16**:751–765.
22. Ivković-Jureković I. Oral allergy syndrome in children. *International Dental Journal* 2015;**65**:164–168.
23. Monaci, L. & Visconti, A. Immunochemical and DNA-based methods in food allergen analysis and quality assurance perspectives. *Trends Food Sci. Technol.* 2010;**21**, 272–283
24. Besler, M. Determination of allergens in foods. *TrAC Trends Anal. Chem*. 2001;**20**, 662–672.
25. Van Hengel, A. J. Food allergen detection methods and the challenge to protect food-allergic consumers. *Analytical and Bioanalytical Chemistry 2007*;**389** 111–118.
26. Downs, M. L. & Johnson, P. Target Selection Strategies for LC-MS/MS Food Allergen Methods. J. AOAC Int. **101**, 146–151 (2018)
27. Vlierberghe, K., Gavage, M., Dieu, M., Renard, P., Arnould, T., De Loose, M. et al. Development and Validation of a Quantitative Method for Multiple Allergen Detection in Food Using Concatemer-Based Isotope Dilution Mass Spectrometry J. AOAC Int. Oline ahead of print (2022). doi: 10.1093/jaoacint/qsac053.
28. Chassaigne, H., Nørgaard, J. V & van Hengel, A. J. Proteomics-Based Approach To Detect and Identify Major Allergens in Processed Peanuts by Capillary LC-Q-TOF (MS/MS). J. Agric. Food Chem. 2007;**55**, 4461–4473.
29. Mills, C., Adel-Patient, K., Bernard, H., De Loose, M., Gillard, N., Huet, A.C. et al. Detection and Quantification of Allergens in Foods and Minimum Eliciting Doses in Food-Allergic Individuals (ThRAll) J. AOAC Int. 2019; **102**:1346-1353.
30. Gavage, M., Van Vlierberghe, K., Van Poucke, C., De Loose, M., Gevaert, K., Dieu, M. et al. High-resolution mass spectrometry-based selection of peanut peptide biomarkers considering food processing and market type variation. Food Chem 2020;**304**:125428–125428.
31. Van Vlierberghe, K., Gavage, M., Dieu, M., Renard, P., Arnould, T., Gillard, N. et al. Selection of universal peptide biomarkers for the detection of the allergen hazelnut in food trough a comprehensive, high resolution mass spectrometric (HRMS) based approach. *Food Chemistry* 2019;**309**:125679.
32. Gavage, M., Van Vlierberghe, K., Van Poucke, C., De Loose, M., Gevaert, K., Dieu, M et al. Selection of egg peptide biomarkers in processed food products by high resolution mass spectrometry. *Journal of Chromatography A 2019*;**1584**:115–125.
33. Van Vlierberghe, K., Gavage, M., Dieu, M., Renard, P., Arnould, T., Gillard, N. et al. Selecting processing robust markers using high resolution mass spectrometry for the detection of milk in food products. *J. AOAC Int. 2022*; **105**: 463–475.
34. Gavage, M., Van Vlierberghe, K., Van Poucke, C., De Loose, M., Gevaert, K., Dieu, M. et al. Comparative study of concatemer efficiency as an isotope-labelled internal standard for allergen quantification. *Food Chem*. 2020;**332**, 127413.

GenEdit : a project to develop and evaluate approaches for detection of organisms modified by new genome editing techniques

Authors :
Frédéric Debode [a] (f.debode@cra.wallonie.be)
Nancy HC Roosens [b] (nancy.roosens@sciensano.be)
Christof Van Poucke [c] (christof.vanpoucke@ilvo.vlaanderen.be)
Yordan Muhovski [a] (y.muhovski@cra.wallonie.be)
Marie-Alice Fraiture [b] (Marie-Alice.Fraiture@sciensano.be)
Katrijn Van Laere [d] (katrijn.vanlaere@ilvo.vlaanderen.be)
Julie Hulin [e] (j.hulin@cra.wallonie.be)
Nina Papazova [b] (nina.papazova@sciensano.be)
Ilse Becue [c] (Ilse.becue@ilvo.vlaanderen.be)
Kevin Vanneste [b] (Kevin.Vanneste@sciensano.be)
Jolien D'aes [b] (Jolien.Daes@sciensano.be)
Gilles Rousseau [f] (g.rousseau@cra.wallonie.be)
Sigrid C.J. De Keersmaecker [b] (Sigrid.DeKeersmaecker@sciensano.be)
Benjamin Dubois [e] (b.dubois@cra.wallonie.be)
Eric Janssen [e] (e.janssen@cra.wallonie.be)
Gilbert Berben [e] (g.berben@cra.wallonie.be)
Marc De Loose [c] (marc.deloose@ilvo.vlaanderen.be)

[a] Walloon agricultural Research Centre (CRA-W), Dept Life Sciences – Biological Engineering Unit, Chaussée de Charleroi 234, 5030 Gembloux, Belgium

[b] Sciensano, Transversal activities in Applied Genomics (TAG), J. Wytsmanstraat 14, 1050 Brussels, Belgium

[c] Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Technology and Food Sciences Unit, Burg. Van Gansberghelaan 115, 9820 Merelbeke, Belgium

[d] Flanders Research Institute for Agriculture, Fisheries and Food (ILVO), Plant Sciences Unit, Burg. Van Caritasstraat 39, 9090 Melle, Belgium

[e] Walloon agricultural Research Centre (CRA-W), Dept Knowledge and valorization of agricultural products – Quality and authentication of agricultural products Unit, Chaussée de Namur 24, 5030 Gembloux, Belgium

[f] Walloon agricultural Research Centre (CRA-W), Dept Knowledge and valorization of agricultural products – Protection, control products and residues Unit, Rue du Bordia, 11, 5030 Gembloux, Belgium

"The research that yielded these results, was funded by the Belgian Federal Public Service of Health, Food Chain Safety and Environment through the contract [RF20/6342]".

Introduction

New genome editing techniques (NGT) allow to induce genetic modifications in plant, animal and microorganism cells. NGT include different techniques (Broothaerts et al., 2021). The most used in food and feed products are techniques that create a double strand break (DSB) in DNA, including site-directed nuclease (SDN), techniques based on clustered regularly interspaced short palindromic repeats (CRISPR), transcription activator-like effector nucleases (TALEN), zinc-finger nucleases (ZFN) and homing endonucleases. All these techniques can lead to (site-specific) mutagenesis and some of them also to cisgenesis, intragenesis or transgenesis.

Available since the eighties, these tools gained in specificity over time. The arrival since 2012 of CRISPR-cas9, characterized by a greater specificity and a more affordable cost, has resulted in a widespread use of this technology. Its application appears endless in agri-food, industrial and medicinal sectors.
Currently, few NGT applications are marketed worldwide: two plants (a soybean with a high oleic acid content and a herbicide-tolerant canola) and one microorganism for release into the environment (bacteria for the fertilisation of agricultural soils) as well as several microorganisms used for contained production of commercial molecules (e.g. biofuels and enzymes). There are, however, about 30 identified applications (plants, animals and microorganisms) in the world at a pre-commercial stage (e.g. for plants : maize, soybean, rice and potato with traits such as herbicide tolerance, fungal resistance, modified oil or starch composition and non-browning properties) in the pipeline that could reach the market in the short term (within 5 years) and a lot of others are at an early stage of development (Parisi and Rodriguez-Cerezo, 2021).

The European Court of Justice (ECJ) decided on July 25th 2018 that organisms modified by NGT like genome editing are considered as genetically modified organisms (GMO) because of the non-natural origin of these modifications.

For a GMO and the derived food and feed products, the European genetic engineering legislation demands event-specific methods for detection, identification, and quantification as part of the authorisation procedure, which is required for marketing in the EU. EU-authorized "classic GMOs" are detectable, identifiable, and quantifiable by polymerase chain reaction (PCR) methods, through the targeting of the stable integration site of "foreign" DNA elements in the genome. As statistically this combination does not occur naturally, it can be considered as a unique identifier. Plants produced by the application of new genomic techniques, however, may lack integrations of any foreign DNA. Moreover, the modifications are often small like the substitution, insertion, or deletion of only a single nucleotide. Hence, the genome sequence of a genome-edited plant may differ only minimally from its parental one.

New detection approaches must therefore be developed for control and evaluated in order to define their limits in pure and mixed products and afterwards lead to a realistic strategy for the distinction between edited and non-edited organisms (or their products). This constitutes a new challenge, as the edited organisms could be difficult, at the technical and analytical level, to distinguish from a natural variation.

Content of the GenEdit project

The proposed research wants to bring answers and solutions concerning the possibilities of detection of organisms modified by new NGT using genomic, metabolomics and proteomic approaches. The laboratories involved in the project, CRA-W, ILVO and Sciensano are all three active in GMO detection and are constituting the National Reference Laboratory (NRL) for GMO detection.

The GenEdit project first proposes a state of the art of the situation (WP1). To achieve this task, a database listing the edited organisms and the genes concerned by the modifications is under construction. Countries of origin will be identified and the status (research, field trial, status towards commercially availability) will be indicated. This is important to observe the trends and to stress where a particular attention is required.

Secondly, we have to know if the available technologies are able to detect organisms produced by NGT.
For this purpose, samples and models are necessary. The samples used in the project will be of three types: genome edited organisms developed by other institutions, models created internally and processed samples that will mimic commercial samples (WP2).
Different genomic methods based on DNA will be tested in WP3 for the detection of gradable modifications. The focus will be on new technologies as well as on techniques easy to implement in enforcement laboratories.
Real-time PCR is the reference technique used in control laboratories. Through proper positioning of the probe, real-time PCR is able to distinguish sequences presenting a difference of more than 3 nucleotides without any problem for first generation GMOs. The probes have generally to be adapted and shortened with the help of different chemistries in order to allow the discrimination of a difference in a single nucleotide position. This also implies to define how the required performance of the methods as the specificity, limit of detection or robustness are affected.
At this level, several members of the project participated to the elaboration of the update of an ENGL guidance document providing recommendations for the detection methods of organisms produced by NGT (ENGL 2022, in preparation).
The High Resolution Melt (HRM) analysis, available on some Real-time PCR machines will be tested. In addition, the use of digital PCR that delivers absolute quantification of the target without any standards and calibration curves will be investigated.
Older techniques generating profiles as PCR RFLP are still interesting and can be modernized thanks to the use of capillary electrophoresis.
Promising technologies as High-throughput sequencing (HTS) will be addressed using different approaches such as amplicon sequencing and enrichment technologies using capture probes. Bioinformatics tools, pipelines and databases, will be necessary to support the tasks and assess the potential diversity of the regions of interest.

Another important question concerns the possible differentiation through the production of specific proteins and metabolites (WP4). We need to know if the analytical strategies based on metabolomics and proteomics are able to detect modifications that will permit to distinguish modified organisms from non-modified ones. The feasibility to set up profiling approaches by the means of LC-HRMS (metabolomics, proteomics) and MALDI-TOF (proteomics) to identify markers for the presence of commodities derived from mutated genotypes will be evaluated. Next, the potential use of more sensitive LC-MS/MS methods to detect these markers known to be expressed in genotypes obtained via NGT will be investigated. As for genomic methods, the performance of these approaches in terms of feasibility, applicability, sensitivity and discriminative power must be assessed.

Finally, it is important to determine how genomic, metabolomic, proteomic and peptidomic profiling techniques fit within a general monitoring strategy that could be considered by the Competent Authorities to carry out their task on Enforcing the Legislation on GMOs (1829/2003 and 2001/18).

References

Broothaerts, W., Jacchia,S., Angers, A., Petrillo, M., Querci, M., Savini, C., Van den Eede, G., Emons, H. (2021). New Genomic Techniques. State-of-the-Art Review. Luxembourg. https://doi.org/10.2760/710056.

Parisi, C., Rodriguez-Cerezo, E. (2021). Current and future market applications of new genomic techniques. Report JRC123830, 49p. ISBN 978-92-76-30206-3.

Court of Justice of the European Union. 2018. Judgment of the Court of Justice of 25 July 2018 in Case C-528/16, Confédération paysanne and Others v Premier ministre and Ministre de l'agriculture, de l'agroal-imentaire et de la forêt, ECLI:EU:C:2018:583.

ENGL 2022 , Gatto, F., Baillie, C.K., Broothaerts, W., Burns, M., Dagand, E., Debode, F., Dobnik, D., Grantiņa-Ieviņa, L., Grohmann, L., Lieske, K., Marchesi, U., Marien, A., Mazzara, M., Papazova, N., Savini, C., van der Berg, J.P., Verginelli, D., Zdeňková, K. Definition of Minimum Performance Requirements for Analytical Methods of GMO Testing – part 2. In finalisation.

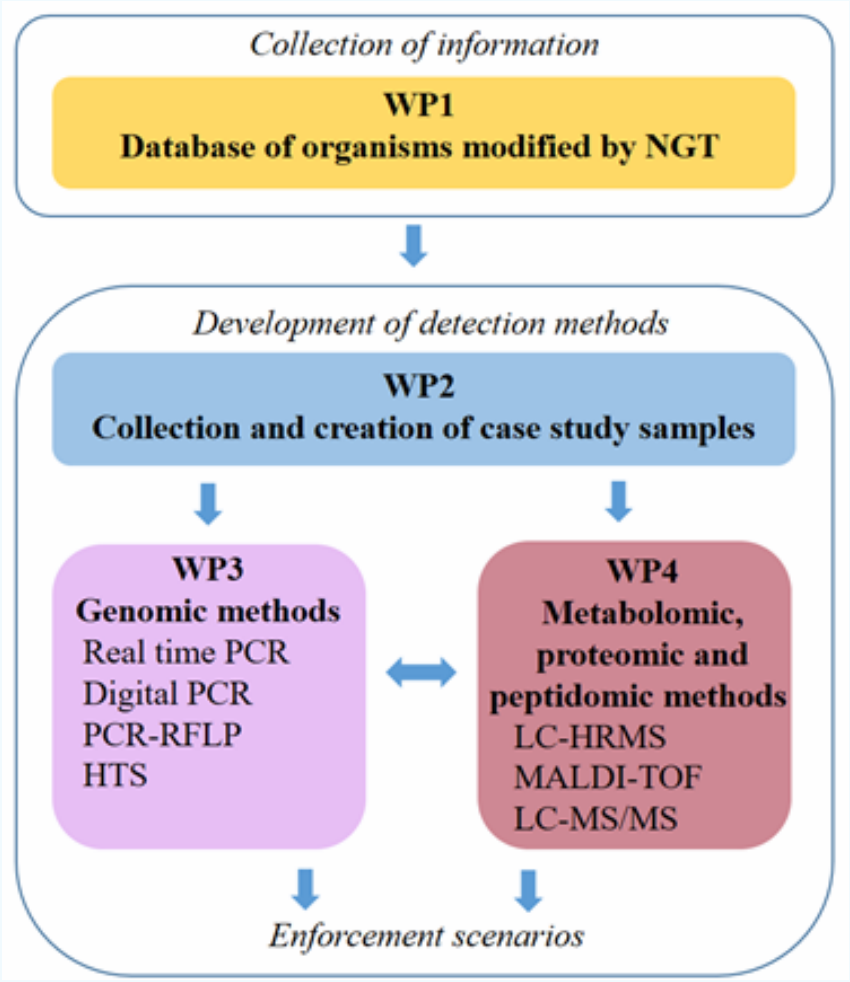


Figure 1: General overview of the work packages (WP) and their interactions in the GenEdit Project

Labinfo: emerging plant toxins

Aline Vanwynsberghe[1], Christelle Robert[1], Els Daeseleire[2] and Nathalie Gillard1

[1] *CER Groupe - Département Santé, Rue du Point du Jour 8, 6900 Marloie, Belgium*

[2] *Instituut voor Landbouw- en Visserijonderzoek (ILVO), Flanders Research Institute for Agriculture, Fisheries and Food Unit Technology and Food, Brusselsesteenweg 370 9090 Melle, Belgium*

Abbreviation list

PA	pyrrolizidine alkaloids
TA	tropane alkaloids
RASFF	Rapid Alert System for Food and Feed
THC	tetrahydrocannabinol
CBD	cannabidiol
EURL	European Reference Laboratory
EFSA	European Food Safety Agency

Nowadays, the consumption of plants and plant-based food is increasing. However, eating a plant or plant-based food is not a synonym for safety nor does it exclude the food being poisonous or harmful. Natural toxins can be found in plants produced by the plant itself (plant toxins) or fungi present on the plant (mycotoxins). The latter might develop either during the growth of the plant or during storage further up in the food chain. Plant toxins or mycotoxins are a matter of food safety as they naturally occur and can be present in food and feed.

Plant toxins are toxic secondary metabolites produced by plants in order to protect themselves against predators, insects, micro-organisms or pests. Plant metabolites are chemical compounds with a high structural diversity which is detailed in Table 1 below (not exhaustive). Toxins can be present in different parts of the plant, such as the leaves, the roots, the fruits or the seeds which means that consumers have to be aware on how to eat or prepare the ingredient for a safe consumption.

As Paracelsus wrote 'Poison is in everything, and no thing is without poison. The dosage makes it either a poison or a remedy'. The consumer and the food safety agency have to keep in mind that the presence of toxins can have detrimental effects on human or animal health.

In order to maintain these emerging risks under control, the European legislation evolves and is currently setting new maximum/action limits for food and feed. For instance, maximum levels in food have already been published for several toxins (see Table 1) and regulations for several other toxin families are under preparation.

In addition to the maximum levels, analytical requirements are laid down in the EU Regulation 401/2006 (currently under revision) which specifies the sampling methods and the analyses methods for official controls regarding mycotoxins. Relating to plant toxins in food, no performance criteria exist yet. It is noteworthy, however, that a separate regulation for plant toxins is foreseen, and that it is considered to harmonize future performance criteria for analytical methods for the determination of mycotoxins and plant toxins.

Besides the evolution of the regulatory levels, it is also crucial to develop in the near future analytical methods that would be easily applicable in routine laboratories. As plant toxins have diversified chemical structures, making their identification, detection and/or quantification quite challenging, it is obvious that no single analytical method will work out for all relevant substances. For instance, at least 12,000 alkaloids exist having as common characteristics a ring structure and a nitrogen atom (Koleva et al., 2012). The next paragraphs will briefly outline the different toxin families.

Pyrrolizidine and tropane alkaloids (PAs and TAs, respectively) are derived from ornithine, a non-essential amino acid. PAs are found ubiquitously in nature as 6,000 plants species have been described to contain it. PAs are present in botanical preparations for food and feed, and a transfer to food of animal origin has been demonstrated (EU Regulation 2020/2040). One notification has been published in 2020 for PAs in chamomile tea in Belgium, implying the recall of the products from the market (Notification 2020.0684). TAs are metabolites present in several plant species, and particularly in weed species. Co-harvesting of TAs-containing weed with food crops such as cereals might be a source of contamination. With the exception of atropine and scopolamine which are regulated (Table 1) only limited toxicological data on TAs are currently available in literature. Two notifications have been published in November 2021 for TAs in infusion (cocoa and cocoa preparations, coffee and tea) and in organic corn flour (cereals and bakery products) in Belgium, implying the recall of the products from the market (Notifications 2021.6059, 2021.6084).

Opium alkaloids are part of the isoquinoline alkaloids family and comprise morphine, codeine and other substances such as oripavine, thebaine and papaverine. These compounds are mainly found in the latex of Papaver somniferum or poppy plants (Figure 1). Poppy seeds are classified into different categories depending on their alkaloid contents: the seeds of varieties with low morphine content intended for culinary purpose, seeds of medium morphine content varieties with a dual role, namely for culinary and industrial purposes, and seeds of varieties with high morphine content, which are intended for industrial or pharmaceutical purpose, and are not edible (Labanca et al., 2018; Mikšík, 2020). The main risk of poppy seed contamination by the alkaloids contained in the latex is during harvesting or damage of the capsule due to an insect. Pharmaceutical cultivation of poppy seeds is mainly for both agents, morphine and codeine, bearing narcotic effects. The content in morphine and codeine is verified in poppy seeds and in bakery products (Table 1) (Knutsen et al., 2018; "Scientific Opinion on the Risks for Public Health Related to the Presence of Opium Alkaloids in Poppy Seeds," 2011). Five notifications for high morphine content have been published in 2020 in Europe having as consequence the withdrawal of the product from the market.

Figure 1 Poppy seed plants (https://alchetron.com/Papaver-somniferum). Papaver somniferum flower and capsule containing the poppy seeds.

Cyanogenic glycosides are not only found in apricot kernels but also in linseed or cassava for which no legal context exists yet when related to products intended for human consumption. It is of note that cyanide in various feeding stuffs is regulated since many decades (Directive 32/2002/EC). Cyanide can have harmful effects by inhibiting oxidative phosphorylation leading to anaerobic energy production, and may cause dyspnea, ataxia, arrhythmia, convulsions, loss of consciousness, decreased respiration and death. There are currently few data for cyanide in raw and processed foods, while also consumption data for cyanogenic glycosides containing foods are few and far between (Schrenk, Bignami, et al., 2019). While a European regulation for food products is under discussion, three RASFF notifications have been published in Belgium since 2020 for linseeds in feed implying the seizure of the lot.

Cannabinoids, and especially tetrahydrocannabinol (THC) are found in the hemp plant Cannabis sativa. Food commodities containing hemp are nowadays in fashion; however, products that can be sold are highly regulated in Europe. In Belgium, it is prohibited to sell food commodities containing plants from list 1 of the royal decree of 31 of August 2021 on the manufacture of and trade of foodstuffs consisting of or containing plants or plant preparations. These provisions also apply to hemp with a THC content of 0.2% or less, although for food commodities containing hemp a derogation to put them legally on the market, might be obtained (cf. FAQ Cannabis). Many culinary options exist with hemp such as hemp seeds, hemp powder, cookies with seeds or pasta made with hemp flour. However, food products containing cannabidiol (CBD) are regulated as novel food. As no authorization has been delivered under the regulation 2015/2283, food products containing CBD are not authorized to be sold (Service Public Fédéral Santé Publique, n.d.).
It is crucial to develop a routine analytical method to detect and quantify Δ9-THC to make sure of none illicit consumption. Regarding feed products, knowledge about the transfer of THC and other linked compounds from feed to animal products is poor. An update of the European legislation is under discussion and likely awaited as it is a growing market. Regarding RASFF notifications, none have been published in Belgium in 2021 nor in 2020 for THC. For CBD, several notifications have in published in the last two years for food and feed products.

Erucic acid is a long chain fatty acid, found in seeds of the species of the Brassicaceae (e.g. rape seed or mustard seed and also seeds from vegetable crops such as kales, cabbages and turnips) and has toxic effect on the heart. There is currently a lack of data for its presence in animal-derived products (meat, milk or eggs) which can be contaminated by the transfer of erucic acid from animal feed. Analytical data on its occurrence in other food commodities such as fine bakery wares or food for infants are needed. However, there are already maximum levels for erucic acid for infant formula and follow-on formula and in other foodstuffs such as vegetable oils and fats, which are laid down in EU Regulations 2019/828 and 2019/1870. One notification has been published in mustard seed oil from Hungary.

Quinolizidine alkaloids are poorly documented. Indeed, occurrence data in food are sparse, as well as knowledge on their transfer from feed to food of animal origin. A legal context is currently lacking for these alkaloids in the EU which might be explained by the paucity of consumption data of lupin and lupin-based foods (Schrenk, Bodin, et al., 2019). One notification for high quinolizidine alkaloid content in bitter lupins from Lebanon has been published in 2021.

Regulation of **hydroxyanthracene derivat**ives (EU regulation 2021/468) has been recently updated due to concern about genotoxic and carcinogenic effects of some substances (Younes et al., 2018). Numerous plants contain hydroxyanthracene derivatives, so it is important to be cautious. The EURL for plant toxins, Wageningen Food Safety Research, is currently developing an analytical method to detect these compounds in food supplements, pills, capsules and drinks sold as laxative. In 2020, one notification for hydroxyanthracene derivatives found in candied green plums from China has been published.

Glycoalkaloids are natural compounds found in Solanaceae flowering plants comprising agricultural crops such as potato, tomato or aubergine plants for instance. The main glycoalkaloids found in potato plant (Solanum tuberosum) are α-solanine and α-chaconine, representing together 95% of the total amount. High concentrations of glycoalkaloids are found in sprout, flowers or leaves and lower levels are present in tubers (Schrenk et al., 2020; Schrenk, Bignami, et al., 2019). It is also important to emphasize that glycoalkaloid contents increase with the peel. Thus, potatoes with high glycoalkaloid concentrations are potatoes with either greening, injury, sprouting or a combination of these parameters. EFSA is calling for data about the identification of good practices to reduce the presence of glycoalkaloids and information on their fate during processing. Based on the current knowledge, EFSA determined the lowest observed adverse reaction level of 1 mg total potato glycoalkaloids/kg body weight per day. No legislation has been published yet for the maximum levels authorized for glycoalkaloids in food or feed but there is a project of recommendation in the EU for the monitoring of glycoalkaloids in potatoes and in products containing potatoes. No RASFF notification has been published for glycoalkaloids.

For the interest of the readers, Wageningen Food Safety Research created a database where a search can be performed based on the genus for a plant and find which contaminants it contains and vice versa. Information can be found on this URL : https://www.wur.nl/upload_mm/d/6/1/0a5ef9aa-85fe-4025-991e-734073939334_Planttox%20database%201.0_online22082012.xlsx.

Table 1 Plant toxins detected in food commodities and related European legislation, legal limits and analytical EURL methods.

Plant toxins	Food commodities	Regulation	Legal limit	EURL method
Pyrrolizidine alkaloids	Herbal infusions: rooibos, anise, lemon balm, camomile, thyme, peppermint, lemon verbena and mixtures exclusively composed of these dried herbs (dried products)	EU 2020/2040 (modifying 1881/2006), applicable from July 1st, 2022	400 µg/kg	EURL-MP-method_002 Pyrrolizidine alkaloids by LC-MS/MS v3
	Other herbal infusions (dried products)		200 µg/kg	
	Tea (Camelia sinensis) and flavoured tea (Camelia sinensis) (dried product)		150 µg/kg	
	Herbal infusions for infants and young children (dried product)		75 µg/kg	
	Herbal infusions for infants and young children (liquid)		1 µg/kg	
	Food supplements containing herbal ingredients including extract		400 µg/kg	
	Pollen based food supplements, pollen and pollen products		500 µg/kg	
	Borage leaves (fresh, frozen) placed on the market for the final consumer		750 µg/kg	
	Borage, lovage, morjoram, oregano (dried)		400 µg/kg	
	Other dried herbs		1000 µg/kg	
	Cumin seeds (seed spices)		400 µg/kg	
Tropane alkaloids	Processed cereal-based foods and baby foods for infants and young children, containing millet, sorghum, buckwheat, maize or their derived products	EU 239/2016 (modifying 1881/2006)	1 µg/kg individual	EURL-MP-method_004 Tropane alkaloids by LC-MS/MS v1
	Unprocessed millet and sorghum	EU 1408/2021 (modifying 1881/2006), applicable from September 1st, 2022	5 µg/kg sum	
	Unprocessed maize with the exception of unprocessed maize intended to be processed by wet milling and with the exception of unprocessed maize for popping.		15 µg/kg sum	
	Unprocessed buckwheat		10 µg/kg sum	
	Maize for popping Millet, sorghum and maize placed on the market for the final consumer Milling products of millet, sorghum and maize		5 µg/kg sum	
	Buckwheat placed on the market for the final consumer Milling products of buckwheat		10 µg/kg sum	
	Herbal infusions (dried product) with the exception of the herbal infusions referred to anise seeds		25 µg/kg sum	
	Herbal infusions (dried product) of anise seeds		50 µg/kg sum	
	Herbal infusions (liquid)		0,20 µg/kg sum	

Opium alkaloids	Whole, ground, milled poppy seeds placed on the market for the final consumer	EU 2021/2142 (modifying 1881/2006), applicable from July 1st, 2022 EU 2014/662 (Good practices)	20 mg/kg	EURL-MP-method_007 Opium alkaloids in food by LC-MS/MS v1
	Bakery products containing poppy seeds and/or derived products thereof		1.5 mg/kg	
Cyanogenic glycosides	Unprocessed whole, ground, milled, cracked chopped linseed	under discussion to split into 2 categories	250 mg/kg-150 mg/kg	NA
	Unprocessed whole, ground, milled, cracked, chopped almonds placed on the market for the final consumer	EU 1237/2017	20.0 mg/lg	
	Cassava (fresh, peeled)	under discussion	50 mg/kg	
	Cassava flour		10.0 mg/kg	
Tetrahydro-cannabinol	Hemp seeds	under discussion	3.0 mg/kg	NA
	Ground hemp seeds (hemp seed powder), (partially) defatted hemp seed (press cake) (hemp seed flour), hemp seed bran		3.0 mg/kg	
	Hemp seed oil		7.5 mg/kg	
Erucic acid	Vegetable oils and fats with the exception of camelina oil, mustard oil, borage oil	EU 1870/2019	20.0 g/kg product	EURL-MP-method_005 Erucic acid by GC-FID v1
	Camelina oil, mustard oil, borage oil		50.0 g/kg product	
	Mustard (condiment)		35.0 g/kg product	
	Infant formulae and follow-on formulae	EU 828/2019	0,4 % of the total fat content	
Quinolizidine alkaloids	Lupin, lupin-derived products	NA	Not defined	EURL + NRLs work of 2021 to develop an analytical method
Hydroanthracene derivatives	Food supplements	EU 468/2021	Prohibited	In development

Knutsen, H. K., Alexander, J., Barregård, L., Bignami, M., Brüschweiler, B., Ceccatelli, S., Cottrill, B., Dinovi, M., Edler, L., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L. (Ron), Nebbia, C. S., Oswald, I. P., Petersen, A., Rose, M., Roudot, A. C., Schwerdtle, T., Vollmer, G., … Vleminckx, C. (2018). Update of the Scientific Opinion on opium alkaloids in poppy seeds. EFSA Journal, 16(5). https://doi.org/10.2903/j.efsa.2018.5243

Koleva, I. I., van Beek, T. A., Soffers, A. E. M. F., Dusemund, B., & Rietjens, I. M. C. M. (2012). Alkaloids in the human food chain - natural occurrence and possible adverse effects. Molecular Nutrition and Food Research, 56(1), 30–52. https://doi.org/10.1002/mnfr.201100165

Labanca, F., Ovesnà, J., & Milella, L. (2018). Papaver somniferum L. taxonomy, uses and new insight in poppy alkaloid pathways. Phytochemistry Reviews, 17(4), 853–871. https://doi.org/10.1007/s11101-018-9563-3

Mikšík, V. & L. V. (2020). The Czech republic the largest producer of breadseed poppy. 28.

Schrenk, D., Bignami, M., Bodin, L., Chipman, J. K., del Mazo, J., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L. (Ron), Leblanc, J. C., Nebbia, C. S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Vleminckx, C., Wallace, H., Benford, D., Brimer, L., Mancini, F. R., … Schwerdtle, T. (2019). Evaluation of the health risks related to the presence of cyanogenic glycosides in foods other than raw apricot kernels. EFSA Journal, 17(4). https://doi.org/10.2903/j.efsa.2019.5662

Schrenk, D., Bignami, M., Bodin, L., Chipman, J. K., del Mazo, J., Hogstrand, C., Hoogenboom, L., Leblanc, J. C., Nebbia, C. S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Schwerdtle, T., Vleminckx, C., Wallace, H., Brimer, L., Cottrill, B., Dusemund, B., … Grasl-Kraupp, B. (2020). Risk assessment of glycoalkaloids in feed and food, in particular in potatoes and potato-derived products. EFSA Journal, 18(8). https://doi.org/10.2903/j.efsa.2020.6222

Schrenk, D., Bodin, L., Chipman, J. K., del Mazo, J., Grasl-Kraupp, B., Hogstrand, C., Hoogenboom, L., Leblanc, J. C., Nebbia, C. S., Nielsen, E., Ntzani, E., Petersen, A., Sand, S., Schwerdtle, T., Vleminckx, C., Wallace, H., Alexander, J., Cottrill, B., Dusemund, B., … Bignami, M. (2019). Scientific opinion on the risks for animal and human health related to the presence of quinolizidine alkaloids in feed and food, in particular in lupins and lupin-derived products. EFSA Journal, 17(11). https://doi.org/10.2903/j.efsa.2019.5860

Scientific Opinion on the risks for public health related to the presence of opium alkaloids in poppy seeds. (2011). EFSA Journal, 9(11). https://doi.org/10.2903/j.efsa.2011.2405

Service Public Fédéral Santé Publique. Questions et réponses concernant l'utilisation de cannabis sativa L. et de cannabinoïdes (comme le cannabidiol) en tant que ou dans les denrées alimentaires. https://www.favv-afsca.be/consumenten/dagelijksleven/andere/basiscannabishennep/_documents/2019_07_12_FAQCannabis_NL_new.pdf

Younes, M., Aggett, P., Aguilar, F., Crebelli, R., Filipič, M., Frutos, M. J., Galtier, P., Gott, D., Gundert-Remy, U., Kuhnle, G. G., Lambré, C., Leblanc, J. C., Lillegaard, I. T., Moldeus, P., Mortensen, A., Oskarsson, A., Stankovic, I., Waalkens-Berendsen, I., Woutersen, R. A., … Wright, M. (2018). Safety of hydroxyanthracene derivatives for use in food. EFSA Journal, 16(1), 1–97. https://doi.org/10.2903/j.efsa.2018.5090

COMMISSION REGULATION (EU) 2020/2040 of 11 December 2020 amending Regulation (EC) No 1881/2006 as regards maximum levels of pyrrolizidine alkaloids in certain foodstuffs

COMMISSION REGULATION (EU) 2016/239 of 19 February 2016 amending Regulation (EC) No 1881/2006 as regards maximum levels of tropane alkaloids in certain cereal-based foods for infants and young children

COMMISSION REGULATION (EU) 2021/1408 of 27 August 2021 amending Regulation (EC) No 1881/2006 as regards maximum levels of tropane alkaloids in certain foodstuffs

COMMISSION REGULATION (EU) 2021/2142 of 3 December 2021 amending Regulation (EC) No 1881/2006 as regards maximum levels of opium alkaloids in certain foodstuffs

COMMISSION RECOMMENDATION (EU) 2014/662 of 10 September 2014 on good practices to prevent and to reduce the presence of opium alkaloids in poppy seeds and poppy seed products

COMMISSION REGULATION (EU) 2017/1237 of 7 July 2017 amending Regulation (EC) No 1881/2006 as regards a maximum level of hydrocyanic acid in unprocessed whole, ground, milled, cracked, chopped apricot kernels placed on the market for the final consumer

COMMISSION RECOMMENDATION (EU) 2016/2115 of 1 December 2016 on the monitoring of the presence of Δ9-tetrahydrocannabinol, its precursors and other cannabinoids in food

COMMISSION REGULATION (EU) 2019/1870 of 7 November 2019 amending and correcting Regulation (EC) No 1881/2006 as regards maximum levels of erucic acid and hydrocyanic acid in certain foodstuffs

COMMISSION DELEGATED REGULATION (EU) 2019/828 of 14 March 2019 amending Delegated Regulation (EU) 2016/127 with regard to vitamin D requirements for infant formula and erucic acid requirements for infant formula and follow-on formula

COMMISSION REGULATION (EU) 2021/468 of 18 March 2021 amending Annex III to Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards botanical species containing hydroxyanthracene derivatives

COMMISSION REGULATION (EC) No 152/2009 of 27 January 2009 laying down the methods of sampling and analysis for the official control of feed

COMMISSION REGULATION (EC) No 401/2006 of 23 February 2006 laying down the methods of sampling and analysis for the official control of the levels of mycotoxins in foodstuffs

DIRECTIVE 2002/32/EC of the European Parliament and of the Council of 7 May 2002 on undesirable substances in animal feed

Workshops & Symposia

The trainings for the approved laboratories organized by the FASFC in co-operation with the National Reference Laboratories are available on the website of the FASFC (www.favv.be > Home > Business Sectors > Laboratories > Seminars & workshops).

The schedule is updated regularly, it is therefore recommended to check the website from time to time.

Other interesting workshops and symposia are mentioned below.

Date	Subject	Place	More information (website)
14 - 16 March 2023	International Conference on GMO Analysis and New Genomic	Berlin (Germany)	https://www.bfr-akademie.de/gmo2023
17-19 April 2023	Food Allergy Forum - 3rd international conference	Amsterdam (Netherlands)	
3 - 5 May 2023	IAFP's European symposium 2023 (International Association for Food Protection)	Aberdeen (Scotland)	https://www.foodprotection.org/
7 - 10 May 2023	11th international Symposium on VTEC 2023	Banff (Canada)	
23 May 2023	International Symposium on Crop Protection, 74th ISCP	Ghent (Belgium)	https://www.ugent.be/bw/plants-and-crops/iscp/en
15-19 June 2023	American Society for Microbiology meeting 2023 (ASM Micro)	Houston (US)	
19-20 June 2023	4th European Food Chemistry Congress	Rome, Italy	
20-22 June 2023	International Symposium Salmonella and Salmonellosis (I3S)	Saint-Malo (France)	
29 June -1 July 2023	ISWAVLD 2023 (International Symposium of the World Asoociaton of Veterinary Laboratory Diagnosticans)	Lyon (France)	https://www.iswavld2023.org/
3-5 July 2023	9th Symposium on Antimicrobial Resistance in Animals and the Environment (ARAE 2023)	Tours (France)	
16-19 July	International Association for Food Protection meeting 2023	Toronto (Canada)	
17-18 August 2023	ICFC 2023: 17. International Conference on Food Contaminants	Budapest (Hungary)	
27-31 August 2023	EuroAnalysis XXI	Geneva (Switzerland)	https://www.euroanalysis2023.ch/index.html
10-14 September 2023	Dioxin 2023 - 43rd International Symposium on Halogenated Persistent Organic Pollutants (POPs)	Liège (Belgium)	www.dioxin2023.org
21 September 2023	Food allergens: regulation, management and detection – 3rd edition	Brussels (Belgium)	
12-13 October 2023	27th conference on Food Microbiology	Brussels (Belgium)	https://www.bsfm.be/en/

NRL NATIONAL REFERENCE LABORATORIES

Labinfo